CHOLESTEROL-LOWERING AND PROBIOTIC PROPERTIES OF SELECTED LACTIC ACID BACTERIA

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การลดคอเลสเตอรอลและคุณสมบัติโพรไบโอติกของแบคทีเรียกรดแลคติกที่คัดเลือกได้

นายอิงครัต กิ่งแก้ว

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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อิงครัต กิ่งแก้ว : การลดคอเลสเตอรอลและคุณสมบัติโพรไบโอติกของแบคทีเรียกรดแลคติกที่คัดเลือกได้. (CHOLESTEROL-LOWERING AND PROBIOTIC PROPERTIES OF SELECTED LACTIC ACID BACTERIA) อ.ที่ปรึกษาหลัก : ศ. ดร.สมบูรณ์ ธนาศุภวัฒน์, อ.ที่ปรึกษาร่วม : ดร.วรรณพ วิเศษสงวน

แบคทีเรียกรดแลคติกทั้งหมด 90 สายพันธุ์ที่แยกได้จากอาหารหมักและเปลือกไม้ถูกคัดกรองฤทธิ์ในการลดคอเลสเตอรอล และพิสูจน์เอกลักษณ์โดยอาศัยลักษณะทางฟีโนไทป์ อนุกรมวิธานเคมี และการวิเคราะห์ลำดับเบสช่วงยีน 16S rRNA สามารถพิสูจน์เอกลักษณ์ได้เป็น *Companilactobacillus farciminis* (5 สายพันธุ์), *C. formosensis* (10 สายพันธุ์), *C. futsaii* (6 สายพันธุ์), *C. pabuli* (4 สายพันธุ์), *Enterococcus thailandicus* (2 สายพันธุ์), *Lactiplantibacillus argentoratensis* (3 สายพันธุ์), *Lactiplantibacillus pentosus* (10 สายพันธุ์), *Lactiplantibacillus plantarum* subsp. *plantarum* (17 สายพันธุ์), *Lactococcus lactis* subsp. *lactis* (2 สายพันธุ์), *Lentilactobacillus buchneri* (8 สายพันธุ์), *Limosilactobacillus fermentum* (2 สายพันธุ์), *P. pentosaceus* (12 สายพันธุ์) และแต่ละสายพันธุ์ของ *En. durans, En. hirae, En. lactis, Lacticaseibacillus paracasei subsp. tolerans, Lacticaseibacillus rhamnosus, Lactococcus lactis subsp. hordniae, Leuconostoc lactis* และ *Pediococcus acidilactici* สายพันธุ์ BCM23-1^T ที่แยกได้จากเปลือกต้นมะขาม มีความใกล้เคียงกับ *Terrilactibacillus laevilacticus* NK26-11^T (98.3 %) สายพันธุ์ BCM23-1^T มีขนาดจีโนม 3.24 Mb ประกอบด้วย 3088 coding sequences (CDS) และมี *in silico* DNA G+C ร้อยละ 37.1 โมล เมื่อวิเคราะห์ค่าร้อยละเฉลี่ยความเหมือนนิวคลีโอไทด์ (ANI) ระหว่าง BCM23-1^T และ NK26-11^T พบว่า มี ค่า ANIb (89.9 %) และ ANIm (90.8 %) และมีค่าดิจิทัลดีเอ็นเอ-ดีเอ็นเอ ไฮบริไดเซชั่น (dDDH) ร้อยละ 40.4 จากข้อมูลของการศึกษาฟีโนไทป์ อนุกรมวิธานเคมี การวิเคราะห์ร้อยละความเหมือนของยีน 16S rRNA การวิเคราะห์แผนภาพวิวัฒนาการ และการวิเคราะห์จากจีโนม บ่งชี้ว่าสายพันธุ์ BCM23-1^T เป็นสปีชีส์ใหม่ในสกุล *Terrilactibacillus* จึงเสนอเป็นแบคทีเรียสปีชีส์ใหม่ชื่อว่า *Terrilactibacillus tamarindi* แบคทีเรียที่คัดเลือกได้ 12 สายพันธุ์มีฤทธิ์ผลิตเอนไซม์ไบลท์ซอลท์ไฮโดรเลส และความสามารถในการนำคอเรสเตอรอลไปใช้ พบว่า *L. plantarum* สายพันธุ์ LM14-2 มีความสามารถในการใช้คอเรสเตอรอลสูงสุดอยู่ที่ร้อยละ 86.07 เมื่อศึกษาคุณสมบัติพื้นฐานของการเป็นโพรไบโอติกของแบคทีเรียกรดแลคติกที่คัดเลือก 12 สายพันธุ์ พบว่าสามารถทนและอยู่รอดได้สภาวะกระเพาะจำลอง นาน 3 ชั่วโมง และลำไส้เล็กจำลอง นาน 5 ชั่วโมง และมีความสามารถยึดเกาะต่อเซลล์ Caco-2 อยู่ที่ร้อยละ 82.46 ถึง 31.16 นอกจากนี้เมื่อนำไปศึกษาความสามารถในการส่งเสริมสุขภาพด้านอื่นๆ พบว่า แบคทีเรียกรดแลคติกที่คัดเลือกได้มีความสามารถในการปรับระดับภูมิคุ้มกัน (IL-12, IFN-γ, hbD2, TNF-α, IL-6 และ NO) นอกจากนี้มีเพียง *Lc. lactis* subsp. *lactis* สายพันธุ์ NH2-7C สามารถผลิตแบคเทอริโอซินที่มีฤทธิ์ในการยับยั้งเชื้อก่อโรค การวิเคราะห์ด้วยข้อมูลจีโนมสามารถระบุได้ว่า แบคเทอริโอซินดังกล่าวคือ ไนซินเอ (nisin A) แบคเทอริโอซิน NH2-7C ถูกนำไปทำกึ่งบริสุทธิ์ผ่านเทคนิค hydrophobic interaction และ เทคนิค cation exchange โครมาโทกราฟี และนำมาศึกษาคุณลักษณะต่างๆ พบว่าแบคเทอริโอซินกึ่งบริสุทธิ์ NH2-7C สามารถทนได้ที่ความร้อน 100 องศาเซลเซียส เป็นเวลา 30 นาที และแสดงฤทธิ์ต้านจุลชีพในสภาวะที่พีเอช 2 ถึง 7 แบคเทอริโอซินกึ่งบริสุทธิ์ NH2-7C ไวต่อเอนไซม์โปรตีเอสเค จากการวิเคราะห์จีโนม *L. plantarum* สายพันธุ์ LM14-2 และ *Lc. lactis* subsp. *lactis* สายพันธุ์ NH2-7C พบว่าทั้งสองสายพันธุ์มีความปลอดภัย ไม่เป็นเชื้อก่อโรคในมนุษย์ และมีคุณสมบัติโพรไบโอจีโนมิก (probiogeomic characteristics)

สาขาวิชา	เภสัชเคมีและผลิตภัณฑ์ธรรมชาติ	ลายมือชื่อนิสิต .....
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KEYWORD: Lactic acid bacteria Fermented foods Tree bark Taxonomy Cholesterol-lowering effects Probiotic health-promoting effects Probiogenomics Whole-genome
Engkarat Kingkaew : CHOLESTEROL-LOWERING AND PROBIOTIC PROPERTIES OF SELECTED LACTIC ACID BACTERIA. Advisor: Prof. SOMBOON TANASUPAWAT, Ph.D. Co-advisor: Wonnop Visessanguan, Ph.D.

Ninety strains of lactic acid bacteria (LAB) isolated from fermented foods and bark of *Tamarindus indica* were screening cholesterol-lowering effects, selected and identified based on the phenotypic, chemotaxonomic characteristics and 16S rRNA gene sequence analysis. They were identified as *Companilactobacillus farciminis* (5 strains), *C. formosensis* (10 strains), *C. futsaii* (6 strains), *C. pabuli* (4 strains), *Enterococcus thailandicus* (2 strains), *Lactiplantibacillus argentoratensis* (3 strains), *Lactiplantibacillus pentosus* (10 strains), *Lactiplantibacillus plantarum* subsp. *plantarum* (17 strains), *Lactococcus lactis* subsp. *lactis* (2 strains), *Lentilactobacillus buchneri* (8 strains), *Limosilactobacillus fermentum* (2 strains), *P. pentosaceus* (12 strains) and each of *En. durans*, *En. hirae*, *En. lactis*, *Lacticaseibacillus paracasei* subsp. *tolerans*, *Lacticaseibacillus rhamnosus*, *Lactococcus lactis* subsp. *hordniae*, *Leuconostoc lactis* and *Pediococcus acidilactici*. Strain BCM23-1^{T} isolated from bark of *Tamarindus indica* was closely related to *Terrilactibacillus laevilacticus* NK26-11^{T} (98.3 %). The draft genome of BCM23-1^{T} was 3.24 Mb in size and contained 3088 coding sequences with an *in silico* DNA G+C content of 37.1 mol%. The values of ANIb, ANIm and digital DNA–DNA hybridization between strain BCM23-1^{T} and *T. laevilacticus* NK26-11^{T} were 89.9, 90.8 and 40.4%, respectively. The results of phenotypic and chemotaxonomic, 16S rRNA gene sequence similarity, phylogenetic tree analysis and whole genome analyses support strain BCM23-1^{T} as representing a novel species of *Terrilactibacillus* for which the name *Terrilactibacillus tamarindi* sp. nov. is proposed. Twelve selected strains exhibited bile salt hydrolase activity (BSH activity) and the ability of cholesterol assimilation. Strain *L. plantarum* LM14-2 showed the greatest cholesterol assimilation ability at 86.07%. From the investigation of fundamental probiotic properties, twelve selected strains could tolerate and survive in the simulated gastric condition for 3 hours and simulated small intestinal condition for 5 hours, and adhere to Caco-2 cells at 82.46 to 31.16%. In addition, all selected strains were evaluated other health-promoting effects. All selected strains showed immunomodulatory effects. Furthermore, only *Lc. lactis* subsp. *lactis* NH2-7C could synthesis bacteriocin containing anti-pathogenic activity. Based on the genomic data, bacteriocin NH2-7C was identified as nisin A. The bacteriocin NH2-7C was partially purified through hydrophobic interaction and cation exchange chromatography and characterized. The partially purified bacteriocin NH2-7C was stable at 100 ◦C for 30 minutes and it showed antimicrobial activity at pH 2 to 7. The partially purified bacteriocin NH2-7C was sensitive to protease-K. Based on the genomic analysis, *L. plantarum* LM14-2 and *Lc. lactis* subsp. *lactis* NH2-7C were safe and they were predicted as non-human pathogen and contained probiogenomic characteristics.

Field of Study:	Pharmaceutical Chemistry and Natural Products	Student's Signature .....
Academic Year:	2021	Advisor's Signature .....
		Co-advisor's Signature .....

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CHAPTER I
INTRODUCTION

Lactic acid bacteria (LAB) are the most commonly used as probiotics, “microorganisms that when administered in adequate amounts confer a health benefit on the host”(Hill et al., 2014), to reduce the level of cholesterol and also promote other health benefits such as modulation of host immune response, and anti-pathogenic activity (Bartley et al., 2010; Joint, 2001; Jones et al., 2013; Somashekaraiah et al., 2019; Tejero-Sarinena et al., 2013). Hypercholesterolemia is a condition that is defined as a very high level of cholesterol in the blood. Since some studies reported that even a small (1%) reduction in serum cholesterol could reduce the risk of coronary heart disease by 2 to 3% (Albano et al., 2018), However, available hypocholesterolemia drugs should not be used for a long term treatment because these drugs may develop some adverse effects (Golomb & Evans, 2008). Thus, probiotics have recently received more attention to prevent hypercholesterolemia.

The possibly interesting mechanisms have been proposed, including removing cholesterol by enhancing fecal bile acid excretion levels by bile salt hydrolase (BSH) enzyme and cholesterol assimilation (Ma et al., 2019). BSH activity has been regarded as a factor related to the cholesterol-lowering activity; hence, BSH activity is also considered as an additional criterion for the selecting probiotics (Miremadi et al., 2014). Numerous studies have interestingly focused on human-, fermented foods-, fruit-, animal- or dairy-derived lactic acid bacteria since some strains of these lactic acid bacteria express bile salt hydrolase activity.

For cholesterol assimilation, it has been suggested that lactic acid bacteria assimilate cholesterol from the medium for their metabolism and growth; consequently, the amount of cholesterol available for absorption in the intestine is reduced. (Lye et al., 2010). The study of Tomaro-Duchesneau et al. (2014) reported that *Lb. reuteri* NCIMB 702656 assimilated about 59.94 ± 7.49 μg/mL of cholesterol in MRS. Also, Albano et al. (2018) demonstrated that *Lb. casei* VC199 could remove approximately 54% of the cholesterol in MRS broth. Thus, it is possible to use

probiotics with cholesterol-lowering effects to reduce serum cholesterol levels in a patient with hypercholesterolemia and prevent hypercholesterolemia in ordinary people. It has become an interesting alternative to drugs available today (Chae et al., 2013). Notably, the effect of LAB is variable even within the same or different species, strain dependence (Kim et al., 2008; Wang et al., 2014).

Recently, the study of Zielinska and Kolozyn-Krajewska (2018) and Sornplang and Piyadeatsoontorn (2016) reported that lactic acid bacteria strains considered to be potential probiotics with interesting activity could be isolated from non-human origins such as foods, fruits, fermented foods, environments and animals. Thus, this study aimed to investigate the cholesterol-lowering effects of LAB isolated from fermented foods and environments, for their potential to deconjugate bile acid via BSH and assimilate cholesterol. Selected strains were evaluated probiotic properties, including viability during gastrointestinal transit, adhesion on Caco-2 cell, immunomodulatory effect, and antimicrobial activity. Furthermore, the antimicrobial compound was characterized. This fundamental information is helpful for further studies and applications.

1.1 The objectives of the study

1. To investigate cholesterol-lowering effects of lactic acid bacteria
2. To study probiotic properties of selected strains
3. To determine immunomodulatory effects of selected strains
4. To screen antimicrobial activity and characterize the antimicrobial compounds of selected strain
5. To evaluate probiogenomic characteristics of selected strains.

CHAPTER II
LITERATURE REVIEW

2.1 Lactic acid bacteria (LAB)

Lactic acid bacteria (LAB) are Gram-positive, typically catalase negative, aerobic or facultative anaerobic bacteria, acid-tolerant, non-spore forming, non-respiring cocci or rods that reside in a diversity of various habitats. The LAB found distinct phyla, namely Firmicutes and Actinobacteria. For the Firmicutes phylum, the most important genera of LAB are *Enterococcus (En.), Lactobacillus (Lb.), Lactococcus (Lc.), Leuconostoc (Ln.), Pediococcus (P.), Streptococcus (S.), Aerococcus (A.), Oenococcus (O.), Carnobacterium (C.), Vagococcus (V.), Tetragenococcus (T.)* and *Weissella (W.)*, which all belong to the order *Lactobacillales* and are low-GC content organisms (31–49 %); on the other hand; the Actinobacteria phylum, LAB belong to the *Bifidobacterium (Bf.)* genus, which have a high-GC content (58–61 %) (Florou-Paneri et al., 2013; Sun et al., 2014). LAB are widespread microorganisms which can be found in various environment such as plants, food products, gastrointestinal tract of human and animal, genitourinary tract of vertebrates, terrestrial and marine animals (Mayo et al., 2008). The LAB can be classified in two metabolic sub-group according to the pathway used to metabolize glucose: homo- and heterofermentative (Basso et al., 2014). For homofermentative LAB, they converted almost 85% of glucose to lactic acid through Embden-Meyerhof-Parnas pathway; whereas, heterofermentative LAB, they converted glucose to lactic acid and other metabolites including, CO_2, acetic acid and/or ethanol through phosphoketolase pathway (Bintsis, 2018).

Lactic acid bacteria play a vital role in the process of food fermentation by inhibiting spoilage/pathogenic bacteria and by producing flavor, aroma, and texture of fermented foods (Mulaw et al., 2019). In addition, interestingly, lactic acid bacteria are the most widely used as probiotics due to numerous studies reported that probiotic lactic acid bacteria promote human health through the inhibition of pathogenic bacteria, modulation of immune system, bile salt hydrolase activity and cholesterol-lowering potential (Capela et al., 2006; Lee & Salminen, 2009; Ozyurt & Ötles, 2014).

2.2 Probiotics

Probiotics described as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (Hill et al., 2014). These microorganisms are mainly belonging to lactic acid bacteria of the *Lactobacillus (Lb.)* and *Bifidobacterium (Bf.)* genera (Table 1) due to Species from the genera *Bifidobacterium* and *Lactobacillus* generally have a satisfactory safety record (Saad et al., 2013; Zukiewicz-Sobczak et al., 2014). However, some genera including *Pediococcus, Leuconostoc*, *Lactococcus lactis,* and *Streptococcus thermophilus* have little information about probiotic properties (Kechagia et al., 2013).

Table 1 Microorganisms used as probiotics

Microorganisms considered as Probiotics		
***Lactobacillus* species**	***Bifidobacterium* species**	**Other LAB**
Lb. acidophilus	*Bf. adolescentis*	*En. faecium*
Lb. casei	*Bf. animalis*	*Lc. lactis*
Lb. crispatus	*Bf. bifidum*	*Ln. mesenteroides*
Lb. curvatus	*Bf. breve*	*P. acidilactici*
Lb. delbrueckii	*Bf. infantis*	*S. thermophilis*
Lb. farciminis	*Bf. lactis*	*S. diacetylactis*
Lb. fermentum	*Bf. longum*	*S. intermedius*
Lb. gasseri	*Bf. thermophilum*	
Lb. johnsonii		
Lb. paracasei		
Lb. plantarum		
Lb. reuteri		
Lb. rhamnosus		

The selection of probiotic requires an essential criterion. This criterion can be roughly divided into four distinct categories: safety, functionality (probiotic properties), technology, and physiology. According to safety assessment, acceptable criteria include human origin, taxonomic identification, non-pathogenic, and absence of virulence, infectivity, toxicity, and transferable antibiotic resistance genes (Sanders

et al., 2010). For the functionality, the probiotics must be able to tolerate to gastrointestinal condition and also adhere and colonize on gastrointestinal epithelial cells (de Melo Pereira et al., 2018). For the technological criteria, the selected probiotics should not have adverse effects on the organoleptic properties of the product. Also, the selected probiotics must tolerate and be viable during product processing and storage (Terpou et al., 2019). Furthermore, some LAB strains can promote host metabolic activities such as bile salt hydrolase activity, cholesterol assimilation, modulation of immune system, and antagonistic activity against pathogen bacteria (Ranadheera et al., 2017; Saarela et al., 2000; Shokryazdan et al., 2017).

Even though, BSH producing lactic acid bacteria and effective cholesterol assimilators are mostly found and isolated in bile acid-rich environment such as human origin (Lye et al., 2010; Tanaka et al., 1999), screening of LAB strains of from foods, fermented foods, and environments (tree barks) for potentially candidate probiotic features have become increased. Higashikawa et al. (2010) reported that LAB isolated from plant origin are more resistant to harsh conditions such as human gastrointestinal tract and also LAB can utilize sugar in plant materials. Thus, if they are studied, examined both of *in vitro* and *in vivo*, particularly in terms of their safety and probiotic properties, they may be an interesting alternative source of LAB from non-human origin (Sornplang & Piyadeatsoontorn, 2016; Zielinska & Kolozyn-Krajewska, 2018).

2.3 Health benefits of probiotics

Probiotics have been associated with a range of various health benefits such as cholesterol-lowering effect, anti-pathogenic activity, and immunomodulation in several studies (Ohashi & Ushida, 2009). Notably, the health promoting characteristics are strain specific, not all strains give desirable health benefits.

2.3.1 Hypocholesterolemic effects

Hypercholesterolemia is a risk factors of cardiovascular disease (CVD) that is the major cause of the mortality (D. R. Labarthe & S. B. Dunbar, 2012; Roth et al., 2011). The coronary arteries are affected by elevated serum cholesterol.

Atherosclerosis, the hardening of the arteries, is a condition that the arteries become narrowed and hardened due to an accumulation of plaque around the artery wall. Plaque is a combination of calcium, cells, fibrous connective tissue, debris, and lipids (cholesterol and fatty acids) that can block the arteries and lead to cardiovascular disease (Marcus, 2013). Therefore, dietary modification, physical activity and functional foods (i.e., probiotics) are attractive choices to reduce the risk of heart diseases and dosage of hypocholesterolemic drugs. (Aronow, 2017; Lichtenstein & Goldin, 2004; St-Onge et al., 2000). In addition, several studies suggested that certain probiotic bacterial strains can assimilate cholesterol. Screening for cholesterol-lowering properties, *in vitro*, has become an essential criterion in the selection of bacterial strains for further *in vivo* probiotic studies (Lin & Chen, 2000; Tahri et al., 1996).

2.3.1.1 Cholesterol

Cholesterol is a waxy, fat-like substance that is only occurred in animal products (Marcus, 2013; Povey, 2016). Cholesterol is not only derived from the diet (exogenous), but also synthesized *de novo* by liver (endogenous) (Arnold & Kwiterovich, 2003). Chemically, cholesterol is an organic compound belonging to the steroid family due to the structure of cholesterol consists of steroid ring and hydroxyl group. Its molecular formula and weight are $C_{27}H_{46}O$ and 386.7 g/mol, respectively as shown in Figure 1 (Kim et al., 2006). Naturally, cholesterol is amphipathic. The polar head of cholesterol is small and consists of 3β-hydroxy group. The rest of the cholesterol molecule is nonpolar and includes a hydrocarbon (isooctyl) tail and four fused rings (the steroid nucleus). Cholesterol is an essential component of cell membranes and modulates membrane fluidity. Also, cholesterol plays a key role as the building block for the biosynthesis including steroid hormones, vitamin D and bile acids (Crockett, 1998; Feleke, 2006).

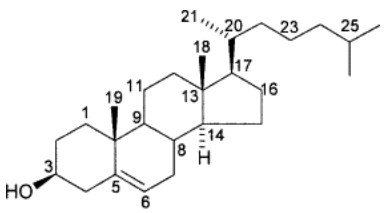


Figure 1 Structure of cholesterol

2.3.1.2 Bile and functions

Bile is a dark green to a yellowish brown aqueous solution whose main constituents include cholesterol, bile acids, phospholipid, and the pigment biliverdin (M. Begley et al., 2006). Bile is mainly produced in the pericentral hepatocytes of the liver, stored and concentrated in the gallbladder interdigestively, and secreted into the first part of the small intestine. Bile plays an important role in fat digestion, which acts as a biological detergent, helping to emulsify and solubilize fats in food. Because of its biological detergent and membranolytic activity, bile is also an antimicrobial compound, a feature considered important for inhibiting the pathogenic colonization in the gut (Tremblay et al., 2017).

The two primary bile acids are cholic acid and chenodeoxycholic acid that are synthesized *de novo* from cholesterol in the liver. After that, the primary bile acid is also metabolized via conjugation to taurine or glycine. The conjugation in the liver increases the solubility of the hydrophobic steroid nucleus; consequently, these molecules are amphipathic and can solubilize lipid to form mix micelles. Bile acids are conserved by a process called enterohepatic recirculation. Bile acids are reabsorbed in the distal part of ileum and colon (Kumar, Nagpal, et al., 2012). Reabsorbed bile acids flow into the portal bloodstream and are taken up by hepatocytes. Later, reabsorbed bile acids are reconjugated and resecreted into bile. Secondary bile acids are formed by gastrointestinal microbiota. Bortolini et al. (1997) informed that “approximately 5% of the total bile acid pool (0.3 g to 0.6 g) per day eludes epithelial absorption and may be extensively modified by the indigenous intestinal bacteria”. One of the main bile acids conversion is deconjugation (Batta et al., 1990).

2.3.2 Mechanisms of cholesterol lowering by probiotics

Numerous mechanisms to lower cholesterol have been proposed in previous researches, such as enzymatic deconjugation of bile salt by probiotics and cholesterol assimilation.

2.3.2.1 Deconjugation of bile acids via bile salt hydrolase enzyme (BSH)

For deconjugation of bile acids, primary bile acids are secreted across the canaliculi to the biliary system into small intestine. The Over than 95% of bile acids secreted in bile are reabsorbed in the distal part of ileum and reabsorbed to the liver (Korpela et al., 1988; Kurdi et al., 2003). Unreabsorbed bile acids flow into the colon and then they are catalyzed by bile salt hydrolase enzyme (BSH) which is produced by probiotics; therefore, the conjugated bile acids are transformed to the deconjugated form. Most deconjugated bile acids are more excreted and found in human feces because of their lower solubility and reabsorption ability, as shown in Figure 2 (Kumar, Nagpal, et al., 2012). Consequently, it causes a reduction in serum cholesterol by increasing cholesterol demand for *de novo* bile acid synthesis to replace the eliminated part. In a homeostatic response, new bile acids are synthesized from cholesterol for maintaining bile acids level, resulting in decreasing of serum cholesterol (Ahn et al., 2003; Bi et al., 2013; Kumar, Ghosh, et al., 2012; Moser & Savage, 2001; Ooi & Liong, 2010). In conclusion, the BSH enzyme performance has a cholesterol-lowering effect. The bile salt hydrolase producing LAB are presented in Table 2.

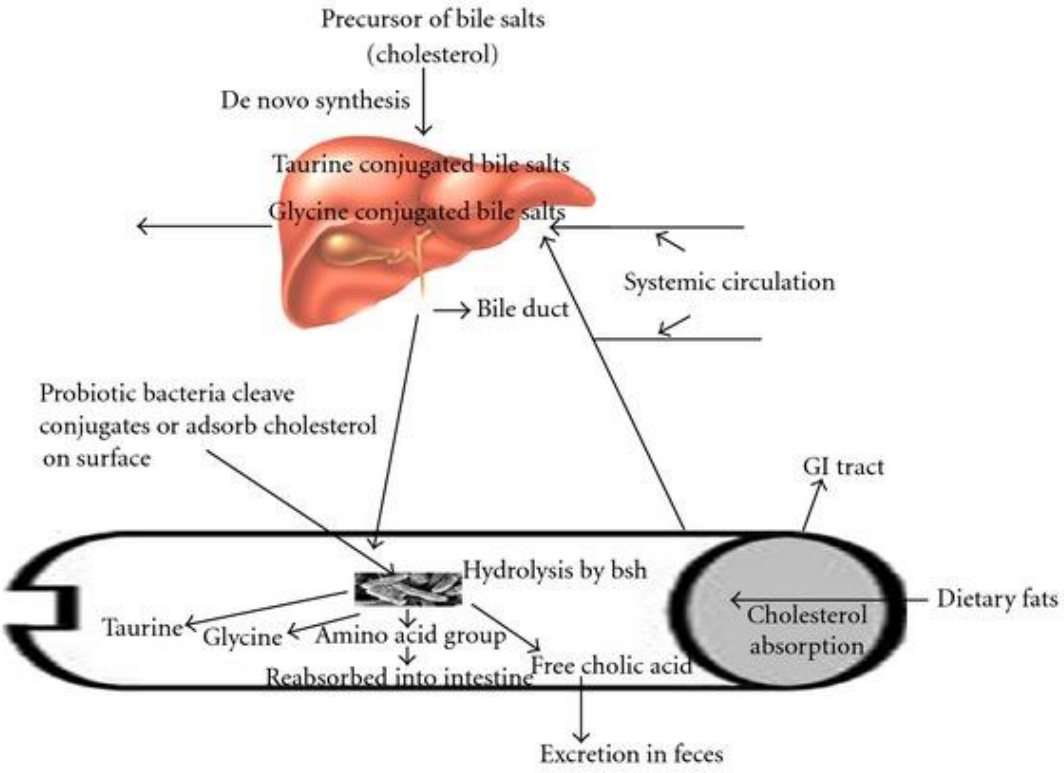


Figure 2 Cholesterol as the precursor for the synthesis of new bile acids and the hypocholesterolemic role of bile salt hydrolase (BSH)

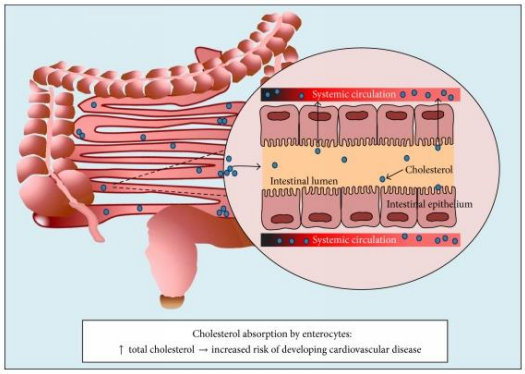
Table 2 Bile salt hydrolase producing LAB

Species	Strain	Source	Reference
Lb. acidophilus	ATCC 4356 BFE 1059	Human Pig feces	Elkins et al. (2001) du Toit et al. (1998)
Lb. brevis	BCCM 18022 UNIVASF CAP 16, UNIVASF CAP 279	Yogurt Goat milk	Elkins et al. (2001) Ferrari et al. (2016)
Lb. rhamnosus	BO3	Boza	Shehata et al. (2016)
Lb. reuter	Iso66	Camel milk	Abushelaibi et al. (2017)
Lb. reuteri	NCIMB 30242	Pig	Hou et al. (2015)
Lc. lactis subsp. lactis	IS-10285	Dadih	Pato et al. (2004)
Lc. garvieae	Iso47	Camel milk	Abushelaibi et al. (2017)
Lb. paracasei	UNIVASF CAP 45, UNIVASF CAP 84	Goat milk	Ferrari et al. (2016)
Lb. delbrueckii subsp. bulgaricus	D11, D14	Dongbei kimchi	Xu et al. (2016)
Lb. fermentum	ATCC 11976	Infant intestine	Elkins et al. (2001)
Lb. gasseri	BCCM 9203	Human	Elkins et al. (2001)
Lb. johnsonii	BFE 1061	Pig feces	du Toit et al. (1998)
Lb. plantarum	GV, GP, SG, OP TGCM 15, TGCM 33 LP96	Fruits Thai fermented food Fermented food	Shekh et al. (2016) Sirilun et al. (2010) Y. F. Liu et al. (2017)
Lb. pentosus	B279, B283, E43, E100, E128	Naturally fermented olives	Argyri et al. (2013)
En. faecium	B20, B21 FAIR-E 154	Stinky soybean Food	Xu et al. (2016) Franz et al. (2001)
En. durans	C12, C5, C3	Rubing	Xu et al. (2016)
P. ethanolidurans	D13	Dongbei kimchi	Xu et al. (2016)
Ln. mesenteroides	V12, V21	Sichuan kimchi	Xu et al. (2016)
Ln. lactis	KC117496	Idli batter	Saravanan and Shetty (2016)
Bf. Longum subsp. *longum*	NRRL B-41409	Adult intestine	Jarocki et al.

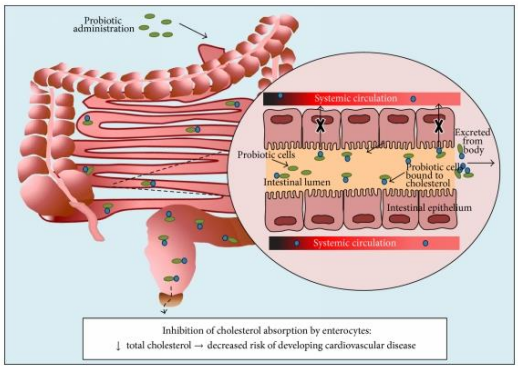
Species	Strain	Source	Reference
			(2014)
Bf. pseudolongum subsp. *pseudolongum*	DSM 20095	Chicken feces	Jarocki et al. (2014)

2.3.2.2 Cholesterol assimilation

Cholesterol assimilation is another mechanism involved in reduction cholesterol levels by probiotics. Due to probiotics can assimilate cholesterol for their metabolism (Bordoni et al., 2013; Liong & Shah, 2005). Consequently, lowering luminal cholesterol levels available for absorption, shown in Figure 3 (Lye et al., 2010; Tomaro-Duchesneau et al., 2014).



(a)



(b)

Figure 3 Schematic representation of probiotic cholesterol assimilation mechanism. (a) Cholesterol absorption by the intestinal. (b) Cholesterol is assimilated by probiotics or bounded to cellular surface

The *in-vitro* study of Abushelaibi et al. (2017) demonstrated that *Lc. lactis* KX881768, *Lb. plantarum* KX881772, *Lc. lactis* KX881782 and *Lb. plantarum* KX881779 can effectively assimilate cholesterol in culture medium. Bordoni et al. (2013) demonstrated that *Bf.* subsp. *infantis* ATCC 15697 assimilated 40 μg/ml and *Bifidobacterium bifidum* MB 109 assimilated 50 μg/ml of cholesterol in MRS broth. Additionally, Shehata et al. (2016) reported that *Lc. lactis* subsp. *lactis* BO37 cells exhibited good cholesterol-removal ability and reached their maximum (43.7%). Michael et al. (2016) stated that *Lb. plantarum* CUL66 exhibited bile salt hydrolase activity and the ability to assimilate cholesterol from culture media (28%).

2.3.3 Immune response

The human immune system is divided into major groups termed innate immune system (non-specific immunity) and acquired immune system (specific immunity). Innate immune system is a mechanism exerting immediate or near-immediate responses to the presence of pathogens. Phagocytic cells, including neutrophils, monocytes, macrophages, and NK cells, enable this first-line defense system against pathogen. However, this first-line defense system largely depends on the number of phagocytic cells and proteins, which then activate the adaptive immune response through the activation of antigen-presenting cells (APCs). On the contrary, adaptive immune system is highly specific and can destroy individual invading pathogens. Besides, a pathogen-specific long-lasting protective memory enables the adaptive immune system to destroy pathogens when reencountered. Lymphocytes, especially B cells and T cells, exert adaptive immune responses by recognizing antigens with their specific receptors (Azad et al., 2018). Probiotics can stimulate, modulate, and regulate immune responses (Jensen et al., 2015; Tsai et al., 2012). The intrinsic properties of probiotic modulate the immune system included elicitation of phagocytic and natural killer (NK) cells, increasing cytokine excretion, and promoting immunoglobulin-secreting cells. (Xiao et al., 2014). Modulation of the regulation of the immune system is likely to play a role in several of the proposed health benefits of probiotics, such as stimulating immune response against various infections and using in specific diseases (immunodeficiency diseases, autoimmune diseases, and (N. Iwabuchi et al., 2012). For these reasons, depending on the purposes and target of the

product, strains that can induce a certain immune response can be selected (Cross, 2002; de Melo Pereira et al., 2018; Gill et al., 2001; Gill et al., 2000; Kirjavainen et al., 1999; Nagafuchi et al., 1999). Probiotics that have been informed as having optimistic effect on immune response are *Bf. lactis, Lb. acidophilus, Lb. casei* Shirota, *Lb. johnsonii*, *Lb. plantarum*, and *Lb. rhamnosus* GG. Notwithstanding, the immune response might enhance when administered of the combination probiotics (Cunningham-Rundles et al., 2000). *Lb. acidophilus, Lb. casei Shirota, Lb. plantarum,* and *Lb. rhamnosus* GG have been reported effective to enhance host defense via interleukine-12 (IL-12) cytokine induction (Christensen et al., 2002; Heufler et al., 1996; N. Iwabuchi et al., 2012; Shida et al., 2011; Tsai et al., 2012). Th1 cells (T helper cell) are involved in the cellular immune response. Th1 cell are characterized by the production TNF-α, IL-12, and IFN- γ. IL-12 is a pro-inflammatory cytokine (Th-1-associated cytokine) and is generated mainly by antigen-presenting cells (APCs) (i.e., macrophage and dendritic cells) in response to microbial stimulation. A major biological function of IL-12 is induction of IFN-γ production (produced by active $CD4^+$ and $CD8^+$ T cells, NK cells, and macrophages). IFN-γ involved host defense against intracellular pathogen infection such as virus, fungal, parasites, and bacteria (i.e., *Salmonella* Typhimurium and *Listeria monocytogenes*) (Price et al., 2007; Romani et al., 1997). For example, IFN-γ can activate phagocytic activity and inducible nitric oxide synthase to synthesize nitric oxide to kill intracellular pathogen and protozoa, and also induce major histocompatibility complex (MHC) class II expression. There have been numerous investigations of LAB inducing NO production (Kmonickova et al., 2012; Korhonen et al., 2001; Surayot et al., 2014). Also, another function of IL-12 is involved antitumor activity such as induction of major histocompatibility complex (MHC) expression on tumor cell and stimulation of cytotoxic activity. The study of Thamacharoensuk et al. (2017) demonstrated that each isolated LAB from Thai fermented foods could induce IL-12 at different levels. Furthermore, an *in vitro* study of Chen et al. (2013) stated that heat-killed multispecies combination of lactic acid bacteria (HMLABs) showed higher induction activities on the production of interleukin-12 with mouse macrophage (RAW 264.7) and also HMLABs and cell walls were able to reduce the *Salmonella* invasion of Caco-2 and mouse macrophage

cells. In addition, IL-6 is a pleiotropic cytokine that is essential to the immune network's comprehensive defense against pathogens and tissue injuries. Clinical investigations of IL-6 inhibitors, namely tocilizumab, demonstrate that their usage is related to an elevated risk of severe and opportunistic infections (Rose-John et al., 2017; Tanaka et al., 2014). *L. paracasei* F19 and *L. plantarum* 2362 supplemented with IL-1β (1 ng/ml) enhanced the level of IL-6 concentration (Reilly et al., 2007). Besides, tumor necrosis factor alpha (TNF-α) is a cytokine that has pleiotropic effects on numerous cell types (Jang et al., 2021). TNF-α protects against bacterial endotoxin, viruses, and parasites, provides enhanced nutrients for immune cells, and favors a proper host response (Galeone et al., 2013). Also, TNF-α can induce tumor cell apoptosis (Pfeffer, 2003), or programmed cell death. In general, TNF-α promotes several cell functions related to immune cell proliferation and adhesion and apoptosis (Aggarwal et al., 2012; Popa et al., 2007). The study of Ashraf et al. (2014) showed that *L. casei* 290 and *St. thermophilus* M5 could potentially stimulate the secretion of TNF-α and IFN-γ.

Moreover, the innate immune system is composed of cellular and humoral components, the latter of which includes antimicrobial peptides present in humans, amphibians, and insects (Dale & Fredericks, 2005; Diamond et al., 2008). In humans, defensins are antimicrobial peptide and serve vital functions in host defense. Especially, β-defensins (BD) are found in the mucosal epithelium and skin (Ganz, 2003). Human (h)BD2 has received attention; hBD2 is induced by inflammation or infection (Ganz, 2003; Harder et al., 2001). Several LAB strains have been shown to stimulate BD expression; hence, enhancing BD expression is likely to prevent infections (Schlee et al., 2008; Zhang et al., 2011).

2.3.4 Antimicrobial activity

Antimicrobial activity is regarded as one of the most beneficial effects of probiotics. Probiotics have several mechanisms to inhibit pathogen such as production of antimicrobial compounds, prevention of the pathogens adhesion, and modulation of the host immune system as shown in Figure 4 (Vieco-Saiz et al., 2019).

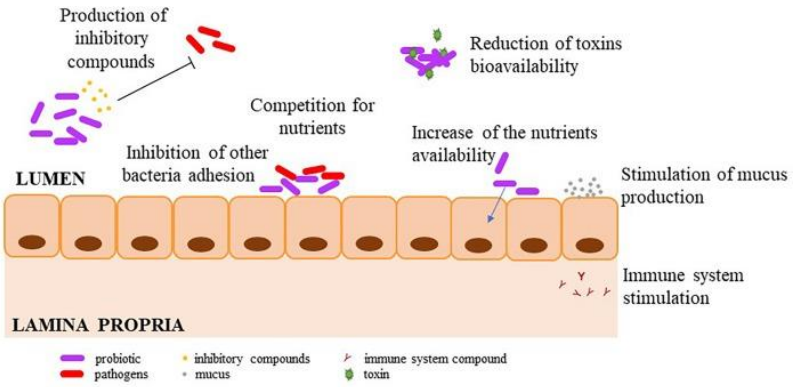


Figure 4 Mechanisms of pathogen inhibition by LAB-probiotics

For the production of antimicrobial substances, study of Islam (2016) also reported that various of anti-pathogenic compounds such as bacteriocin-like compounds, ethanol, organic acids, diacetyl, acetaldehydes, hydrogen peroxide (H_2O_2), reuterin, and peptides were synthesized by probiotics. Interestingly, peptides and bacteriocins are mostly involved against pathogen by increasing the membrane permeability of the target cells; consequently, the membrane is depolarized, ultimately, cell death (Simova et al., 2009). The study of Tejero-Sarinena et al. (2013) investigated that the influence of probiotics on the survival of *Sa.* Typhimurium and *Clostridium difficile* in an *in vitro* and postulated that probiotics inhibit enteric pathogens by short-chain fatty acids (SCFAs), such as acetic, propionic, butyric and lactic acids. Additionally, SCFAs also help to maintain pH in lumen (Kareem et al., 2014). In addition, *Lb. reuteri*, which reside in gastrointestinal tract of humans and animals, can produced broad-spectrum antimicrobial compound called reuterin. Reuterin can inhibit the growth of several pathogenic bacteria as *Cl.* spp., *Es.* spp., *Salmonella (Sa.), Shigella (Sh.)* spp., *Staphylococcus (St.)* spp., *Proteus (Pt.)* spp., and *Pseudomonas (Ps.)* spp. (Axelsson et al., 1989; Talarico et al., 1988).

2.3.4.1 Bacteriocin

Bacteriocins are ribosomally synthesized peptides by various groups of bacteria including lactic acid bacteria. Bacteriocins have either bactericidal or bacteriostatic activity (Prudencio et al., 2015). Interfering of cell wall and the membrane of target organisms, either by inhibiting cell wall biosynthesis or causing

pore formation, consequently resulting in death and/or growth inhibition, are the proposed mechanisms of bacteriocins. In addition, bacteriocins have several desirable characteristics such as are recognized as safe (GRAS), nonactive or cytotoxic substance to eukaryotic cells and degraded by digestive enzymes (protease) (da Costa et al., 2019). For these reasons, bacteriocins are recommended in several studies as alternative to existing preservatives in the food products such as meat and dairy products, canned foods, instant soups, also for therapeutic applications in the medical field (Kaya & Simsek, 2019). For instance, pediocin PA-1 is produced by *P. acidilactici* that has potential to extend the shelf-life of food products particularly by inhibiting the growth of *Listeria monocytogenes* (Kumariya et al., 2019).

(I) Classification of bacteriocins

Bacteriocins are classified majority into two distinct categories based on the structural and characteristic shown in Table 3 (Cotter et al., 2005).

Table 3 Classification scheme of bacteriocins

Classification	Remarks	Examples
Class I		
Lanthioine-containing bacteriocin (lantibiotics)	Includes both single- and two-peptide lantibiotics.	**Single peptide:** nisin, mersacidin, and lacticin 481 **Two-peptide:** lacticin 3147 and cytolysin
Class II		
Non-lanthionine-containing bacteriocins	**Heterogeneous class of small peptides;** - **subclass a:** pediocin-like - **subclass b:** two-peptide - **subclass c:** cyclic - **subclass d:** non-pediocin single linear peptides	**Class IIa:** pediocin PA1, leucocin A **Class IIb:** lactacin F **Class IIc:** enterocin AS48, reuterin 6 **Class IId:** lactococcin A, divergicin A
Bacteriolysins		
Non-bacteriocin lytic proteins	Large, heat-labile proteins, often murein hydrolases	Lysostaphin, enterolysin A

(II) Anti-pathogenic activity of bacteriocin

Nowadays, the studies on antimicrobial activity of bacteriocins produced by probiotic-LAB have become increased, since they have proven effectiveness in inhibition the pathogenicity of food-borne pathogens. However, Gram-negative bacteria are naturally resistant to the bacteriocin that produced from Gram-positive bacteria because of outer layer membrane (lipopolysaccharide) that plays a key role as a potential barrier (Cao-Hoang et al., 2008; Gyawali & Ibrahim, 2014). The research of Casey et al. (2004) demonstrate that *Lb. salivarius* subsp. *salivarius* DPC6005 and *Lb. salivarius* subsp. *salivarius* M7·2 isolated from porcine intestinal origin exhibited the effective inhibition of gram-negative and gram-positive bacteria such as *Sa.* Typhimurium BAA 185, *Escherichia coli* O157:H7 AR12900, *Li. innocua* DPC 5073, *Staphylococcus aureus* DPC 5246. Simova et al. (2009) reported that bulgaricin BB18, produced by *Lb. bulgaricus* BB18 isolated from traditional Bulgarian dairy products, exhibits antimicrobial activity to gram-negative and gram-posive such as *Sa.* Typhimurium FVK781, *Helicobacter pylori* HPK78, *Listeria* (*Li.*) *monocytogenes* C12, *Clostridium difficile* TCN16, and *Bacillus subtilis* 1A95. In addition, both gram-negative and gram-positive bacteria are inhibited by purified Bac7293A and Bac7293B that produced by *Weisslla (W.) hellenica* BCC7293 isolated from Nham (Thai fermented pork sausage) (Woraprayote et al., 2015). The study of Stern et al. (2006) found that purified bacteriocin OR-7, produced from *Lb. salivarius* NRRL B-30514, showed antimicrobial activity to *Campylobacter (Ca.) jejuni* NCTC 11168. Furthermore, bacteriocin DY4-2 produced by *Lb. plantarum* DY4-2 isolated cutlassfish (*Trichiurus lepturus*), that showed inhibitory activity against gram-negative and gram-positive bacteria (Lv et al., 2018). Therefore, the isolation of potential bacteriocin producing LAB from non-human origin, evaluation of probiotic properties and the safety for using in food and pharmaceutical application are attractively investigated.

(III) Bacteriocin characteristics

Molecular weight and amino acid sequence

The molecular mass and amino acid sequencing of antimicrobial peptides are used as tools for bacteriocins classification and identification. Elegado et al. (1997)

and Ennahar et al. (2000) reported that the molecular mass of normal purified antimicrobial peptides are about 2.5 to 6.5 kDa. Purified bacteriocins produced from *Enterococcus faecium* ICIS 7 was estimated to be about 6.5 kDa (Vasilchenko et al., 2018). The research of Woraprayote et al. (2015) reported that the molecular weight of Bac7293A and Bac7293B produced from *W. hellenica* BCC7293 were 6,249 and 6,489 Da, respectively. The approximate molecular mass of bacteriocin DY4-2 synthesized from *Lb. plantarum* DY4-2 is 1,465 Da (Lv et al., 2018).

Furthermore, its N-terminal sequencing always be used to identify or classify the type of antimicrobial peptides. Lv et al. (2018) reported that bacteriocin DY4-2 contains 13 amino acids, HAQIGMTMNGSFR. reported that bacteriocin DY4-2 contains 13 amino acids, HAQIGMTMNGSFR. Simova et al. (2009) stated that the molecular mass of bulgaricin BB18 produced from *Lb. bulgaricus* BB18 is around 4.2 kDa. Bulgaricin BB18 was consisted of 31 amino acids (KIYRGNVGHCGKSTVDWGTAIGNGNNAASFL) and also it was classified into class IIa non-lantibiotics bacteriocins. Also, the research of Stern et al. (2006) reported that purified bacteriocins OR-7 produced from *Lactobacillus salivarius* NRRL B-30514 is about 5,123 Da. The amino acid sequencing of bacteriocin OR-7 is N'-KTYYGTNGVHCTKNSLWGKVRLKNMKYDQNTTYMGRLQDILLGWATGAFGKTF-C' and classified into class IIa.

(IV) Factors affecting the antimicrobial activity of bacteriocin

Temperature

The effect of various temperatures on antimicrobial activity of antimicrobial peptides has been evaluated. Simova et al. (2009) studied the sensitivity of antimicrobial peptide that produced by *Lb. bulgaricus* BB18 to heat treatment. After heat treatment at 100 °C for 60 min, and 115 °C for 15 min, antimicrobial activity of antimicrobial peptide was slightly decreased. Antimicrobial activity of bacteriocin DY4-2 produced by *Lb. plantarum* DY4-2 show the stability to heating at 100 °C for 30 min and 121 °C for 30 min. After heat treatment at 121 °C for 30 min, the activity of antimicrobial peptide is slightly decreased (Lv et al., 2018). Stern et al. (2006) studied the sensitivity of bacteriocin OR-7 to heat treatment. After heat treatment at

37 °C for 2 h, 37 °C for 24 h and 90 °C for 20 min, antimicrobial activity of bacteriocin OR-7 was not decreased. Furthermore, the research of Yi et al. (2016) investigated that bacteriocin MN047 produced from *Lb. crustorum* MN047 is mostly resistant to heating at 100 °C for 10,20, and 30 min. Summarily, all of the results can possibly be concluded that antimicrobial peptide possess the stability to heating at 100 °C for 10-30 min.

pH

Since bacteriocins are peptide compound; therefore, the study of pH is crucial to evaluate antimicrobial activity. A few studies have investigated the effect of pH on anti-pathogenic activity of antimicrobial peptides. The research of Woraprayote et al. (2015) reported that the activity of purified bacteriocin Bac7293A and Bac7293B synthesized from *W. hellenica* BCC7293 decreased upon the exposure to high pH (alkalinity condition). *Lb. plantarum* DY4-2 isolated from cutlassfish (*Trichiurus lepturus*), produced bacteriocin DY4-2 that had the stability over a pH range from 2 to 9 and is retained more than 70% activity even at high pH (pH 10 and pH 11) (Yi et al., 2016). Stern et al. (2006) studied the sensitivity of bacteriocin OR-7 to various pH condition (3.0 to 10). Antimicrobial activity of bacteriocin OR-7 was stable at pH values ranging from 3.0 to 9.1 but became inactive at pH 10. Additionally, the research of Simova et al. (2009) stated that the antimicrobial peptide produced by *Lb. bulgaricus* BB18 exhibited the good stability to a wide range of pH from 5 to 8.

Enzymes

Several enzymes including lipases, amylase, and proteases that usually presented in foods and gastrointestinal tract were used to evaluate the effect of these enzymes on anti-pathogenic activity of antimicrobial peptides. Anti-pathogenic activity of antimicrobial peptide produced from *Lb. bulgaricus* BB18 was inactivated when this antimicrobial peptide was exposed to α-chymotrypsin, papain, pepsin, proteinase K, protease XI, protease XIV and trypsin, whereas α-amylase and lipase were not influence anti-pathogenic activity (Simova et al., 2009). Purified bacteriocin Bac7293A and Bac7293B synthesized from *W. hellenica* BCC7293 was totally inactivated by proteolytic enzymes such as α-chymotrypsin, pepsin, trypsin,

proteinase K; on the other hand, it was insensitive to α-amylase and lipase (Woraprayote et al., 2015). Purified bacteriocin OR-7 synthesized from *Lb. salivarius* NRRL B-30514 was inactivated by proteolytic enzymes such as β-chymotrypsin, proteinase K, and papain; on the other hand, it was insensitive to lysozyme and lipase (Stern et al., 2006). In addition, anti-pathogenic activity of bacteriocin DY4-2 synthesized from *Lb. plantarum* DY4-2 was entirely inactivated after treatment with papain, pepsin but partially inactivated by nutrase (Lv et al., 2018).

CHAPTER III
MATERIALS AND METHODS

3.1 Media, chemicals and equipments

1. 0.22 µm pore size filter Millipore (MA, USA)
2. 24-well tissue culture plates (Corning, USA)
3. 25% Trypsin (Gibco-Invitrogen, USA)
4. 3-(4,5-dimethylthiazol-2-yl) -2,5 diphenyltetrasolium bromide (MTT) (Sigma, Germany)
5. 37% Hydrochloric acid (HCl) (Merck, Germany)
6. 96-well tissue culture plates (Corning, USA)
7. Acetic acid (CH_3COOH) (Merck, Germany)
8. Agar (Difco, USA)
9. Anaeropack TM-MicroAerobic and Anaerobic gas generator kit (Mitsubishi, Japan)
10. API 50 CH (bioMérieux, USA)
11. Autoclave, Model : HA-3D (Hirayama, Japan)
12. Calcium carbonate ($CaCO_3$) (Merck, Germany)
13. Calcium Chloride ($CaCl_2$) (Merck, Germany)
14. Cell line : RAW 264.7 cells
15. Cell line : Caco-2 human colon carcinoma cells (ATCC HTB-37)
16. Cell line : THP-1 cells
17. Centrifuge (Sartorius, Germany)
18. Centrifuge 5810 R (Eppendorf, Germany)
19. Cholesterol PEG-600 (Sigma, USA)
20. CO_2 incubator (NAPCO 6000, Thermo Scientific, USA)
21. Conical tube 15 and 50 mL (Corning, USA)
22. Counter (Fisher Scientific, USA)
23. De Man-Rogosa-Sharpe (MRS) or Lactobacilli MRS broth (Difco, USA)
24. Dimethyl sulfoxide (DMSO) (Fisher Scientific, India)
25. Dulbecco Modified Eagle medium (DMEM) (Gibco-Invitrogen, USA)
26. Ethanol (EtOH) (Merck, Germany)

27. Fetal bovine serum (FBS) (Gibco-Invitrogen, USA)
28. Gel Electrophoresis (Model : GE-100, China)
29. Hemocytometer (Hausser Scientific, USA)
30. Hexane (C_6H_{14}) (Merck, Germany)
31. Incubator, Model : BE600, Memmert, Germany
32. Laminar flow hood (Model : BV-126, ISSCO, Thailand)
33. Medium 199 (M199) (Gibco-Invitrogen, USA)
34. Methanol (MeOH) (Merck, Germany)
35. Microscope CHS model (Olympus, Japan)
36. Multi-Detection Microplate Reader (BioTek Synergy HT, USA)
37. O-phthaldialdehyde (OPA) (Sigma, USA)
38. Oxgall or bovine bile (Sigma, USA)
39. PCR Authorized Thermal Cycler (Bio-Rad Laboratories, California)
40. PCR DNA fragment extraction kit (Geneaid Biotech, Taiwan)
41. Penicillin-Streptomycin (Gibco-Invitrogen, USA)
42. Phase contrast microscopy (ZEISS Primo Star, USA)
43. Potassium hydroxide (KOH) (Fisher, USA)
44. Roswell Park Memorial Institute medium number 1640 (RPMI 1640) (Gibco-Invitrogen, USA)
45. Skimmed milk (Difco, USA)
46. Sodium chloride (NaCl) (Sigma-Aldrich, USA)
47. Sodium hydroxide (NaOH) (Merck, Germany)
48. Sodium salt of taurodeoxycholic acid (TDCA) (Sigma, USA)
49. Speed vacuum (Rotational Vacuum Concentrator Rvc 2-18, Germany)
50. Sulfuric acid (H_2SO_4) (Merck, Germany)
51. Syringes (NIPRO, Thailand)
52. Tissue culture flask 25 and 75 cm (Corning, USA)
53. HumanTNF-alpha DuoSet ELISA (R&D systems a biotechne brand, USA)
54. Human IL-6 DuoSet ELISA (R&D systems a biotechne brand, USA)
55. Amberlite-XAD N16 (Sigma, USA)
56. Sp-sepharose (Sigma, USA)
57. Trifluoroacetic acid (Merck, Germany)

58. Enzymes Kit (Sigma, USA)
59. Wizard Genomic DNA Purifcation kit (Promega Corporation, USA)

3.2 Methodology

3.2.1 Experimental plan

Lactic acid bacteria (LAB) isolated from fermented foods and tree barks. All isolated LAB were identified, screened for bile salt hydrolase activity. The LAB strains which exhibit bile salt hydrolase were selected for further studies. The selected LAB strains were examined probiotic properties. The antimicrobial compound involving in the inhibition of pathogenic bacteria were partially purified and characterized. The selected strains were also analyzed the probiogenomic characteristics from the genomic data. The experimental plan of this study is presented in the Figure 5.

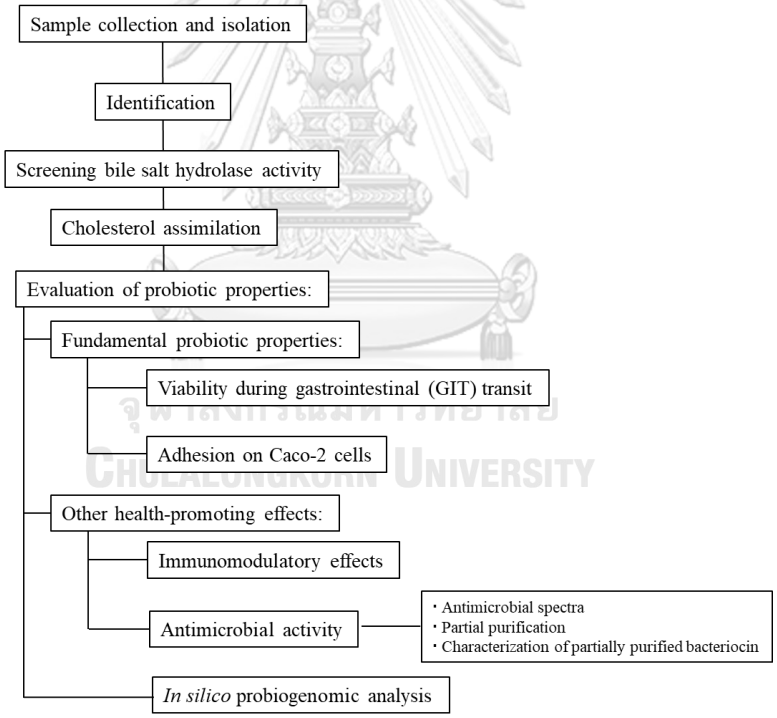


Figure 5 Experiment plan in this study

3.2.2 Collection of samples and isolation

In this study, fermented food products and tree barks samples were collected in Thailand. For isolation, 10 g or 10 ml of each sample was suspended and homogenized in 90 mL of De Man Rogosa Sharpe (MRS) broth (De Man et al., 1960)

and incubated at 30 °C for 72 h under aerobic condition. After incubation period, one loopful of culture broth was streaked on MRS agar supplemented with 0.3% (w/v) calcium carbonate ($CaCO_3$) and incubated under the same conditions. The colonies surrounding with clear zone were selected and picked up for purification based on different morphology. This procedure was repeated until obtained the pure cultures. The cultures were kept at -20 °C in 40% (v/v) glycerol and lyophilized with 10% (w/v) skim milk for further studies.

3.3 Identification

3.3.1 Phenotypic characteristics

After 48 hours of cultivation on MRS agar plate and incubation at 30 ºC, colony appearance, cell shape, cell arrangement, catalase activity, and Gram staining were determined. Physiological and biochemical characteristics include growth in 4%, 6%, and 8% (w/v) NaCl, growth at the temperatures 15 ºC, 30 ºC, and 45 ºC, growth at different pH (2.0-9.0, using relevant buffer), nitrate reduction, gas production, aesculin hydrolysis, arginine hydrolysis, and acid production from carbohydrates (conventional and API 50 CH (bioMérieux) were examined as described by Tanasupawat et al. (1998). Isomer of lactic acid was enzymatically analysed as previously describied by Okada et al. (1978). The hierarchical cluster analysis for grouping the isolates using SPSS for Window version 22.0 was performed based on the phenotypic characteristics.

3.3.2 Chemotaxonomic characteristics

3.3.2.1 Cell wall composition

Cells were grown in MRS broth at 30 ºC for 48 h, harvested by centrifugation at 10,000 rpm for 5 min and then washed twice with 0.85% NaCl solution. The amino acids in the cell wall was detected by TLC technique (Hasegawa et al., 1983). Briefly, cells pellet was hydrolyzed by using 0.1 ml of 6N HCl (hydrochloric acid) and heated by autoclaving at 121 ºC for 15 min. After cooling, 1:1 of hydrolysate was spotted onto a cellulose TLC plate. The TLC plate was developed with the solvent system of methanol-water-6N HCl-pyridine (80:26:4:10 v/v) for 3 h. After the second developing, the TLC plate was sprayed with ninhydrin spray reagent and heated at

100 ºC for 2 min to visualize the spot. *meso*-diaminopimelic acid (DAP) was used as the standard.

3.3.2.2 Cellular fatty acid analysis

Strains were cultivated in MRS broth at 30 ºC for 4 days. Gas chromatography (GC) was used for cellular fatty acid analysis following the instruction of Microbial Identification System (MIDI) Sherlock version 6.0 (Kämpfer & Kroppenstedt, 1996; Sasser, 1990). Dry cell (40 mg) was suspended in 0.1 ml of reagent 1 (sodium hydroxide 15 g, methanol 50 ml and milli-Q water 50 ml) and vigorously mixed for 5-10 seconds with a vortex mixer. The solution will be heated at 100 ºC for 5 min, mixed and heated again at 100 ºC for 25 min. After cooling, 2 ml of reagent 2 (6 N-HCl 65 ml, methanol 55 ml) was added to the test tube, mixed and heated at 80 ºC for 10 min. The mixture was added with 1.25 ml of reagent 3 (n-hexane 50 ml, methyl-tert-butyl ether 50 ml) and mixed for 10 min. The upper layer was transferred to a new tube and added with 3 ml of reagent 4 (sodium hydroxide 1.2 g, milli-Q water 100 ml). The tube was mixed for 5 min and transferred 2/3 of the sample to a GC vial. The cellular fatty acids were analyzed by using gas chromatography.

3.3.3 Genotypic characteristics

3.3.3.1 16s rRNA gene sequencing

The representative strains of each group were selected to sequence their 16S rRNA gene sequences. The 16S rRNA gene sequences of isolates were amplified using polymerase chain reaction (PCR) technique with primer 20F (5'-AGTTTGATCCTGGCTC-3') and 1530R (5'AAGGAGGTGATCCAGCC-3'). PCR products were sequenced using a DNA sequencer (Macrogen, Korea) with universal primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3'), 518F (5'CCAGCAGCCGCGGTAATACG-3'), 800R (5'-TACCAGGGTATCTAATCC-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3') ((Lane, 1991)).,The sequence similarity values between the isolates and their related reference isolates were calculated using the EzBiocloud tool (Yoon et al., 2017). MEGA 7 constructed a phylogenetic tree using the neighbor-joining (NJ) approach (Kumar et al., 2016;

Saitou, 1987). A bootstrap analysis with 1000 replications was used to determine the confidence values for each branch in the phylogenetic tree (Felsenstein, 1985). The identified sequences were deposited in DDBJ (DNA Data Bank of Japan, Mishima, Japan).

3.3.3.2 Whole-genome sequencing

The genomic DNA of strain BCM23-1T was extracted from cells grown in MRS broth after incubating at 30 °C for 48 h and was purified by the method of Saito and Miura (1963). The Nanodrop ND-2000 UV-Vis spectrophotometer and the Qubit 3.0 fluorometer (Thermoscientific) were used to measure the purity and concentration of the extracted genomic DNA. At Omics Science and Bioinformatics Center, Chulalongkorn University, library preparation and sequencing of the strain LM14-2 were performed by Nextera XT DNA prep kit and Illumina Miseq sequencer. Quality of raw reads was checked using FASTQC software (Galaxy Version 1.1.5). Adaptors and poor-quality reads were removed using Trim Galore (Galaxy Version 0.6.3), filtered reads were used as an input for Unicycler, genome assembly program (Galaxy Version 0.4.8.0). The genomic quality and contamination were evaluated by CheckM (Parks et al., 2015). The sequence similarity values between the strains and their related reference strains were computed using the EzBiocloud tool (Yoon et al., 2017). Then, the average nucleotide identity (ANI) and the digital DNA-DNA hybridization (dDDH) values were analyzed using JSpeciesWS web server tool (Richter & Rosselló-Móra, 2009; Richter et al., 2016) and the Genome-to-Genome Distance Calculator (GGDC 2.1) using the BLAST+ method with formular 2 (Meier-Kolthoff et al., 2013). The ANI of >95% and DDH of >70% are considered to be the same species (Kim et al., 2014). A phylogenetic tree based on whole-genome sequence was constructed by using TYGS web server (https://tygs.dsmz.de/) (Meier-Kolthoff & Göker, 2019). Furthermore, genomic circular map was constructed using CGView Server (Grant & Stothard, 2008).

3.3.3.3 Gene prediction and functional annotation

The draft genome was annotated by using the DFAST sever (Tanizawa et al., 2018), Rapid Annotation Server Technology (RAST) (Aziz et al., 2008), PATRIC

(Davis et al., 2020), the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) (Tatusova et al., 2016). Antibiotic resistance genes were determined using the Comprehensive Antibiotic Resistance Database (CARD; https://card.mcmaster.ca) (Alcock et al., 2020), and ResFinder web-based tool (Bortolaia et al., 2020). The pathogenicity was predicted by PathogenFinder web-based tool (Cosentino et al., 2013) and plasmid was detected by PlasmidFinder (Carattoli et al., 2014). The PHAge Search Tool Enhanced Release (PHASTER) was used to identify and annotate putative prophage sequences (Arndt et al., 2016). The Identification of carbohydrate-active enzymes was performed using the dbCAN meta server (https://bcb.unl.edu/dbCAN2/blast.php) with HMMER: biosequence analysis with profile hidden Markov models (version: 3.3.2), and all data generated in dbCAN were based on the family classification from the CAZy database (http://www.cazy.org/) (Cantarel et al., 2009; Zhang et al., 2018). The biosynthesis of antimicrobial peptides gene clusters was detected and visualized by BAGEL4 (BActeriocin GEnome mining tool; http://bagel4.molgenrug.nl) (van Heel et al., 2018). Genes responsible for virulence and undesirable characteristics may be identified using publicly available databases and manually inspected to confirm its identity and function. Precautions are required in the interpretation of the findings since genes involved in survival and adaptation should not be treated as virulence genes for non-pathogenic bacteria. The search using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (https://www.kegg.jp) (Kanehisa et al., 2016) for the pathways and genes.

Determination of cholesterol-lowering effects

3.4 Screening bile salt hydrolases (BSH) activity

The BSH activity was determined as described by Shehata et al. (2016) with minor modification. 20 µl of the overnight culture broth was spotted on MRS agar supplemented with 0.5% (w/v) taurodeoxycholic acid sodium salt (TDCA) and 0.037% (w/v) calcium chloride. Plates was incubated anaerobically at 37 °C for 72 h. The halos around colonies or white opaque colonies were indicated the bile salt hydrolase activity of bacteria. The non-modified MRS was used as control. The BSH-producing LAB were be selected for evaluation of probiotic properties.

3.5 Cholesterol assimilation in MRS

The capability to assimilate cholesterol was evaluated following the method of Tomaro-Duchesneau et al. (2014) with minor modification. MRS broth was supplemented with Cholesterol-PEG 600 at a final concentration 100 µg/ml. The MRS broth without supplementation of cholesterol stock solution was used as control. A 1% (v/v) of cell suspension was inoculated in and incubated anaerobically at 37 °C for 24 h. At the end of incubation period, the cell free supernatant (CFS) was harvested by centrifugation at 4,000x*g* for 20 min at 4 °C. The residual cholesterol content was determined. In brief, 100 µl of CFS was mixed with100 µl of KOH (33%, w/v) and 200 µl of 95% ethanol, vortexed for 1 min, incubated at 37 °C for 15 min, and cooled to room temperature. Thereafter, 200 µl of MilliQ water and 500 µl of hexane were added into the mixture solution and vortexed for 1 min. The mixture was allowed to settle until separation of the two layers. One hundred microliters of the upper hexane layer was collected and evaporated under nitrogen. After that, 200 µl of *o*-phthalaldehyde reagent (50 mg OPA in 100 ml glacial acetic acid) was added and gently shaken for 1 min. After incubation at room temperature for 3 min, 50 µl of sulphuric acid was added and gently mixed followed by resting for 10 min at room temperature before measuring the absorbance at 550 nm using UV-spectrophotometer. A standard curve of absorbance versus cholesterol concentrations was generated using the cholesterol concentrations: 0, 3.125, 6.25, 12.5, 25, 50, 75, 100, and 125 µg/ml. The cholesterol concentration was read off a standard curve prepared using the cholesterol stock solution. The ability of probiotics to assimilate cholesterol in MRS was reported as the percentage of cholesterol removed at each incubation interval as follows:

$$\text{Cholesterol assimilated } (\mu g/ml) = [\text{Cholesterol } (\mu g/ml)]_{0\,h} - [\text{Cholesterol } (\mu g/ml)]_{24\,h}$$

$$\%\,\text{Cholesterol assimilated} = \left[\frac{\text{Cholesterol assimilated } (\mu g/ml)}{\text{Cholesterol } (\mu g/ml)_{0\,h}} \right] \times 100$$

3.6 Evaluation of probiotic properties

Fundamental probiotic properties

Preparation of LAB cell suspension

For the studies of probiotic properties, the cell suspension was prepared following the procedure of Pithva et al. (2014). The selected strains were cultivated twice in MRS broth at 30 ºC for 24 h. After incubation period, the cells were collected by centrifugation at 14,000 rpm for 10 min at 4 ºC, washed twice with phosphate-buffered saline (PBS; 0.1 M, pH 7.2, containing 0.85% (w/v) NaCl), and resuspended in phosphate buffer (0.1 M, pH 7) to get cell suspension of $A_{600} = 1$ and 10^9 CFU/ml.

3.6.1 Viability during gastrointestinal (GIT) transit

In vitro assessment of characteristics for viability in the transit of gastrointestinal tract were assessed using an *in vitro* model simulated gastric fluid and intestinal fluid as described by Minekus et al. (2014) with minor modification. Briefly, the cell suspension was mixed with simulated gastric fluid (SGF) containing pepsin 2000 U/ml and incubated anaerobically for 3 hours at pH 3 and 37 °C. The samples were collected at 0 (initial time) and 3 (gastric-emptying time) hours of incubation time for viably bacterial enumeration. After the incubation time, the gastric chyme was transferred, mixed with simulated small intestinal fluid (SIF) containing pancreatin (based on trypsin activity at 100 U/ml) and 10 mM of bile, and incubated anaerobically for 5 hours at pH 7 and 37 °C. The samples were collected at 0 (initial time) and 5 (small intestinal-emptying time) hours of incubation time viably bacterial enumeration. The number of viable LAB was quantified using a serial 10-fold dilution and spot plate technique. The *L. rhamnosus* GG was used as control. The viable bacteria were reported as logarithms of colony-forming units per milliliter ($\log_{10}$CFU/ml).

3.6.2 Adhesion assay

The human intestinal epithelial cell line, Caco-2 cell line was used to assess the adhesion capacity of selected LAB strains following the method of Han et al. (2017) with minor modification. Caco-2 cells were routinely cultured in Dulbecco's Modified Eagle's Medium (DMEM) high glucose supplemented with 10% (v/v) fetal bovine serum (FBS), and 1% (v/v) penicillin-streptomycin. The Caco-2 cells were

cultured in tissue flask at 37 ºC in 5% CO_2. For the adhesion test, the Caco-2 cells were treated with trypsin then incubated at 37 ºC in 5% CO_2 for 5 min, to elute adherent Caco-2 cells from tissue flask. The Caco-2 cell concentration were adjusted to 5×10^5 cell/ml and seeded into 24-well tissue culture plates. The tissue plates were incubated at 37 ºC in 5% CO_2 until the Caco-2 cells attained a confluent-differentiated monolayer state. The Caco-2 cells were washed twice in PBS before the experimental use. The cell suspension was harvested by centrifugation at 14,000 rpm for 10 min at 4 ºC and re-suspended again in DMEM without antibiotics. Next, 1 ml of each cell suspension was inoculated into well then incubated for 90 min at 37 ºC in 5% CO_2. After incubation period, Caco-2 cells were washed three times with PBS to rule out the unbounded cells then cells were eluted by addition of 1 ml of 0.05% Triton-X100 solution. The number adherent bacteria were enumerated by spot-plate technique on MRS agar then incubated at 37 ºC for 48 h. The *L. rhamnosus* GG was used as control. The adherent ability of selected strains was calculated according to the following equation as previously described by Alp and KuleaŞan (2020);

$$\text{Adhesion percentage (\%)} = \frac{N_t}{N_0}\times100$$

Where; N_t = the log CFU of adherent bacterial cells to the Caco-2 cells

N_0 = the log CFU of inoculated bacterial cells

Other health-promoting effects

3.6.3 Immunomodulation effects of selected strains

The selected strains were prepared and evaluated for immunomodulatory effects following the method of Hosaka et al. (2021).

3.6.3.1 Preparation of sterilized lactic acid bacteria powder

Each selected strain was inoculated into MRS broth and incubated for 24 hours at 120 rpm with shaking at 30°C. Following sterilizing the culture media at 100°C for 20 minutes, the bacteria were collected by centrifugation at 1,000 rpm for 10 minutes. To prepare sterilized lactic acid bacteria powder, the bacteria were washed with sterile distilled water and then lyophilized. For preparing the test sample, the test sample was suspended in PBS at a concentration of 200 µg/ml.

3.6.3.2 Cell culture and cell differentiation

RAW264.7 cells were cultured in Dulbecco's modified Eagle medium (DMEM) (SIGMA) supplemented with 5% FBS and 0.2% PS at 37°C in a 5% CO_2 incubator. Caco-2 cells were provided by Professor Shinichi Yokota, Sapporo Medical University School of Medicine. Cultures were grown in Dulbecco's modified Eagle medium supplemented with 5% FBS and 0.25% PS in a 5% CO_2 incubator at 37°C. THP-1 cells were cultured in RPMI 1640 medium supplemented with 10% FBS and 0.2% PS in a 5% CO_2 incubator at 37°C.

Caco-2 cells ($1.5x10^5$ cells) were seeded on cell culture inserts (Falcon, 24-Well Hanging Inserts 0.4 μm) and cultured for 72 hours. After 72 hours, the media containing 5 mM sodium butyrate was replaced, and the cells were incubated for 96 hours to induce differentiation. Differentiated cells were evaluated by transepithelial electrical resistance (TEER) using Millicell-ERS (Merk), and differentiated cells with TEER values greater than 400 $\Omega x cm^2$ were used. THP-1 cells were seeded on a multi-well plate (24 well, Falcon) and incubated for 3 days in media containing 100 ng/ml cholecalciferol (Vitamin D_3) and 10 nM phorbol12-myristate13-acetate (PMA) to differentiate into macrophage-like cells. Following differentiation, Caco-2 and THP-1 cells were co-cultured in Transwell.

3.6.3.3 Measurement of NO production

Nitric oxide (NO) production was determined as described by Yang et al. (2018). RAW264.7 cells were suspended in DMEM medium (5% FBS + 0.2% PS) at a concentration of 3 $x10^5$ cells/ml, seeded in each 24-well multi-well plate and incubated at 37 °C in a 5% CO_2 incubator for 24 hours. The test sample was added to stimulate the cells (final concentration of 20 μg/ml). PBS was used as a negative control, and LPS (10 μg/ml) (Fujifilm Wako) as a positive control. After 24 hours of stimulation, the supernatant was collected and centrifuged at 12,000 rpm for 20 minutes and evaluated by Griess reaction, as reported by Baek et al. (2015). 100 μl of each Griess reagent, medium supernatant sample, and 1.56-100 μM sodium nitrite standard solution was added to 96-well microplates and incubated for 20 minutes at room temperature. The absorbance at 550 nm was measured in a microplate reader,

and the nitrite concentration in the medium supernatant was calculated using a calibration curve obtained from the sodium nitrite standard solution.

3.6.3.4 Intestinal Immunity Model

An *in vitro* intestinal immune model was simulated by co-culture cell culture inserts (apical side) and multi-well plates (basal side). The test sample was suspended in RPMI 1640 medium was added to the apical side (final concentration 20 µg/ml), and the cells were stimulated in a 5% CO_2 incubator at 37°C for 48 hours. After incubation, the basal side of the medium was collected, and after centrifugation at 12,000 rpm for 20 min, the supernatant was collected to remove foreign substances. For IL-12 and IFN-γ, proteins were precipitated by adding a 25% volume of 100% TCA to the culture medium supernatant sample. The precipitates were washed with acetone to remove TCA and dissolved in 1X sample buffer for protein enrichment after heat treatment at 100 °C for 2 min. The proteins were separated by SDS-PAGE performed according to Laemmli (1970), and the target proteins were detected by Western blot according to the method reported by Towbin et al. (1979). Calibration curves were prepared with known concentrations of IL-12 standard and IFN-γ standard to calculate the production of IL-12 and IFN-γ. Production was corrected by measuring β-actin as an endogenous control. For hBD2, unenriched medium supernatant was measured by the Dot blot, and the amount of hBD2 production was corrected from the total protein by CBB staining. The values were evaluated relative to the no-stimulation test section with PBS.

3.6.3.5 The immunomodulatory effects of Lc. lactis subsp. lactis NH2-7C

The strain NH2-7C was analyzed by Biodiversity Research Centre, Research and Development Group for Bio-Industries, Thailand Institute of Scientific and Technological Research (TISTR), Pathum Thani 12120, Thailand. For the heat-killed cell preparation, strain NH2-7C was cultivated twice in MRS broth and incubated at 30 °C for 24 h. The bacterial cells were collected by centrifugation at 10,000 rpm for 10 min and heated at 85 °C for 10 min. Then, the lysate was filtered through 0.22 µm and lyophilized before the test. The supernatant of strain NH2-7C was collected, heated at 85 °C for 10 min, filtered through 0.22 µm and lyophilized before the test.

(I) THP-1 cell culture

THP-1 cells were maintained in complete RPMI-1640 medium. Complete medium consisted of 500 ml RPMI 1640 medium, 50ml FBS, 5ml penicillin/streptomycin and 5ml L-glutamine. Cells were incubated at 37°C, 5% CO_2 and 100% humidity. A suspension of 500 µl of THP-1 cells was seeded in a 24 well plate at a density of $1x10^5$ cells/ml with phorbol 12-myristate 13-acetate (PMA) at a concentration of 60 ng/ml in each well, and incubated at 37ºC in 5% CO_2 and 100% humidity for 48 h. The differentiated cells were checked and then the medium re-suspended to the same volume without PMA and incubated for a further 24 h. Heat-killed cells and supernatant of strain NH2-7C (500 µl) were added together with lipopolysaccharide (LPS, 1 µg/ml) and without LPS and then incubated for another 24 h. The supernatants were collected and stored at -20 °C until analyzed by Enzyme-Linked Immunosorbent Assay (ELISA).

(II) Determination of TNF-α and IL-6 from THP-1 cells

TNF-α and IL-6 levels were measured following the manufacturer's instructions of a Human TNF-alpha DuoSet ELISA (R&D systems a biotechne brand, USA) and human IL-6 DuoSet ELISA (R&D systems a biotechne brand, USA), respectively.

3.6.4 Screening of antimicrobial activity

3.6.4.1 Cultures and Cultivation

Strains were cultivated on MRS agar and incubated at 30 °C for 48 h and the strains were propagated twice in MRS broth at 30 °C for 18 h. The *L. sakei* JCM 1157 was used as indicator strains and the other indicator strains were used for the study of antimicrobial spectra. The appropriate medium and condition are shown in Table 4.

Table 4 The appropriate medium and condition for indicator strains

Indicator strain	Testing condition		
	Medium	**pH**	**Temp. (°C)**
Gram-positive bacteria:			
Bacillus circulans JCM 2504^{T}	TSB	7.3	30
B. subtilis JCM 1465^{T}	TSB	7.3	30
B. cereus	TSB	7.3	30
Enterococcus faecalis JCM 5803^{T}	MRS	6.5	37
En. faecium JCM 5804^{T}	MRS	6.5	37
Lactobacillus plantarum ATCC 14917^{T}	MRS	6.5	30
Lb. sakei JCM 1157^{T}	MRS	6.5	30
Lactococcus lactis ATCC 19435^{T}	MRS	6.5	30
Leuconostoc mescenteroides JCM 6124^{T}	MRS	6.5	30
Listeria inocua ATCC 33090^{T}	TSB	7.3	37
Li. monocytogenes ATCC 19115	TSB	7.3	37
Micrococcus luteus MIII	TSB	7.3	37
M. luteus NBRC 12708 *(Kocuria rhizophila)*	TSB	7.3	30
Pediococcus dextrinicus JCM 5887^{T}	MRS	6.5	30
P. pentosaceus JCM 5885	MRS	6.5	30
Staphylococcus aureus ATCC 23235	TSB	7.3	37
Methilcillin-resistant *S. aureus* DMST 20635	TSB	7.3	37
S. aureus ATCC 25923	TSB	7.3	37
S. aureus ATCC 6538	TSB	7.3	37

S. aureus Cowan I	TSB	7.3	37
S. aureus DMST 6512	TSB	7.3	37

Table 4 The appropriate medium and condition for indicator strains

	Testing condition		
Indicator strain	**Medium**	**pH**	**Temp. (°C)**
Gram-positive bacteria:			
Streptococcus agalactiae 1611	TSB	7.3	37
St. gordonii DMST 35778	TSB	7.3	37
St. iniae SI 1810	TSB	7.3	37
St. mutans DMST 18777	TSB	7.3	37
St. pyogenes DMST 17020	TSB	7.3	37
St. suis NaH	TSB	7.3	37
St. suis P1/7	TSB	7.3	37

Table 4 The appropriate medium and condition for indicator strains

Indicator strain	Testing condition		
	Medium	**pH**	**Temp. (°C)**
Gram-negative bacteria:			
Aeromonas hydrophila B1 AhB1	TSB	7.3	37
Campylobacter coli NCTC 11353^{T*}	BHI	7.4	42
Escherichia coli ATCC 25922	TSB	7.3	37
E. coli O157:H7	TSB	7.3	37
E. coli F18	TSB	7.3	37
E. coli ATCC 35401	TSB	7.3	37
E. coli JCM 1093	TSB	7.3	37
H. pylori ATCC 43504*	BHI + 5% Sheep blood	7.4	37
H. pylori 3875*	BHI + 5% Sheep blood	7.4	37
H. pylori BK 364*	BHI + 5% Sheep blood	7.4	37
Pseudomonas aeroginosa ATCC 27853^{T}	TSB	7.3	37
Salmonella typhimurium ATCC 13311^{T}	TSB	7.3	37
Vibrio algenolyticus Va	TSB + 1.5% NaCl	7.3	37
V. harveyi AQVH 01	TSB + 1.5% NaCl	7.3	37
V. parahaemolyticus DMST 26792^{T}	TSB + 1.5% NaCl	7.3	37

V. parahaemolyticus with AHPND toxin plasmid 1691	TSB + 1.5% NaCl	7.3	30
V. parahaemolyticus without AHPND toxin plasmid 1681	TSB + 1.5% NaCl	7.3	30
V. vulnificus 1809	TSB + 1.5% NaCl	7.3	30
Yeast & Mold:			
Candida albicans ATCC 10231	TSB	7.3	25
Candida albicans ATCC 90028	TSB	7.3	25

*, These indicators were incubated in microaerobic condition.

3.6.4.2 Determination of antimicrobial activity

Selected strains were cultivated at 30 ºC for 18 h in MRS broth under aerobic condition. Culture supernatant was harvested by centrifugation at 14,000 rpm for 10 min at 4 ºC. The cell-free supernatant (CFS) was adjusted to pH 6.5 ± 0.1 with 1 M NaOH (Hu et al., 2017; Lv et al., 2018; Zhang et al., 2019). After that, the neutralized cell-free supernatant (NCFS) was filtered through 0.22 pore-size sterile filter prior to the antimicrobial assay. The antimicrobial activity was determined by the spot-on-lawn method as described by Ennahar et al. (2001). The antimicrobial assay was be divided into 2 parts including qualitative assay and semi-quantitative.

For qualitative assay, 10 µl of the aliquot of NCFS was be spotted directly onto the prepared indicator lawn in soft agar (0.7% (w/v); top layer) that overlaid on solidified agar (1.5% (w/v); bottom layer). Culture medium used in this test is depend on indicator strain (Table 4). The indicator lawn was prepared by 5 ml of soft agar seeded with an overnight culture of each indicator strains approximately 10^7 CFU/ml (Lima et al., 2007). The plates were dried for 30 min, and then incubated at the appropiate conditions for each indicator strain. The effective NCFSs showed a transparent inhibition zone of the indicator strain were selected for further semi-quantitative assay (Casey et al., 2004; van Reenen et al., 1998).

For semi-quantitative assay, two-fold serial dilution of effective NCFS was prepared in sterile distilled water in a sterile 96-well microtiter plate. Next, 10 µl of each two-fold serial dilution of effective NCFS was spotted directly on indicator lawn that overlaid on solidified agar as described above. The plates were dried for 30 min and incubate at same condition. After incubation period, the antimicrobial activity was recorded and reported in arbitrary activity units per milliliters (AU/ml) which was defined as the reciprocal of the highest two-fold serial dilution showing a transparent inhibition zone of the indicator strain according to the following formula;

$$\text{The antimicrobial activity (AU/ml)} = (2^N) \times 100$$

Where; AU : Arbitrary Unit

N : The highest two-fold serial dilution showing a transparent inhibition zone of the indicator strain.

3.6.4.3 Partial purification of antimicrobial compound

(I) Time course bacteriocin production

The antimicrobial compounds production of selected strain was following the method of Woraprayote et al. (2015) with modifications. An overnight selected strain was inoculated into 200 ml of MRS broth then incubated at 30 ºC. Samples were taken and recorded at 4 h intervals for 48 h, and the bacterial growth and the changes in pH were detected using UV-Visible (600 nm) and digital pH meter, respectively. The antimicrobial activity (AU/ml) was evaluated by the critical dilution spot-on-lawn assay.

(II) Partial Purification

The antimicrobial compound was partially purified as described by Woraprayote et al. (2015). An overnight selected strain was inoculated into 1 L of MRS broth at 30 ºC for 20 h under aerobic condition. The CFS was collected by centrifugation at 8,000×*g* for 15 min at 4 ºC. Briefly, antimicrobial peptide was extracted using a series of hydrophobic interaction chromatography (Amberlite XAD-16 polymeric resin) and fast flow cation-exchange chromatography (SP-sepharose resin) with stepwise gradient from 0.25 to 1.0 M NaCl in 20 mM sodium phosphate, pH 5.7. All fractions were collected and determined for the antimicrobial activity by spot-on-lawn technique.

3.6.4.4 Protein determination

Protein concentration was determined by Lowry (1951) using bovine serum albumin (BSA) as standard.

3.6.4.5 Characterization of partial purified bacteriocin

(I) The effect of various enzymes on antimicrobial activity

Sensitivity of bacteriocin to various enzymes was evaluated as described by Hu et al. (2017) with minor modifications. The partially purified bacteriocin was incubated with 1.0 mg/ml final concentration of various enzymes as trypsin, α-chymotrypsin, pepsin, protease-K, lipase and amylase in the appropriate buffer at 37 ºC for 5 h. At the end of incubation period, the solution mixture was heated at 100 ºC for 5 min to deactivate the enzymes and adjusted to pH 6.5. The residual antimicrobial

activity was determined by spot-on-lawn method using *Lb. sakei* JCM 1157^T as an indicator strain. The sample without enzyme treatment was used as a control.

(II) The effect of chemicals on antimicrobial activity

Organic solvents

Effect of chemicals, including organic solvents and surfactants, on the antimicrobial activity of bacteriocin was studied as previously described of KaraoĞLu et al. (2003). Various organic solvents such as acetonitrile, ethanol and isopropanol were added to partially purified bacteriocin solution at 1:1 ratio. All samples were thoroughly mixed and kept at room temperature for 5 h before antimicrobial test. The residual antimicrobial activity was determined by spot-on-lawn method using *Lb. sakei* JCM 1157^T as an indicator strain. Untreated partially purified bacteriocin solution and organic solvent with equal volume of sterile ultrapure water were used as controls.

Chemicals

Effect of chemicals on antimicrobial activity of partially bacteriocin was determined by incorporating non-ionic (Triton X-100, Tween 20, Tween 80), anionic (sodium dodecyl sulphate) surfactant, EDTA and urea following the procedure of Pinto et al. (2009). All agents were added to partially purified bacteriocin to yield the final concentration of 1% (w/v). All samples were incubated at 30 °C for 5 h before antimicrobial test. The residual antimicrobial activity was determined by spot-on-lawn method using *Lb. sakei* JCM 1157^T as an indicator strain. Untreated purified bacteriocin and chemicals at this concentration in sterile distilled water were used as controls.

(III) The effect of pH on antimicrobial activity

The impact of pH to antimicrobial activity of partially purified bacteriocin was determined following the method of Ahn et al. (2017) with slight modifications. The partially purified bacteriocin was adjusted to various pH 2, 3, 5, 7, 9, 11 and 13 with 5 N of HCl or NaOH, then incubated at 37 ºC for 4 h. After incubation period, the sample was neutralized to pH 6.5 and tested residual antimicrobial activity by spot-on-lawn method using *Lb. sakei* JCM 1157^T as an indicator strain. The untreated sample was used as a control.

(IV) The effect of temperature on antimicrobial activity

The thermostability of partially purified bacteriocin was determined as described by Lim (2015). The partially purified bacteriocin was incubated at 100 ºC for 10, 20, and 30 min, and by autoclaving at 121 ºC for 15 min, respectively. At the end of incubation period, the partially purified bacteriocin was immediately cooled in ice bath and adjusted to pH 6.5. The residual antimicrobial activity was determined by spot-on-lawn method using *Lb. sakei* JCM 1157^T as an indicator strain. The untreated sample was used as a control.

3.7 Statistical analysis

All experiment were done in triplicate and reported as the mean ± standard deviation (SD). Results was analyzed by ANOVA (Analysis of variance) by SPSS 22.0 software. Duncan's Multiple Range Test (DMRT) was used for comparison for mean values at a significant level of $P \leq 0.05$.

CHAPTER IV
RESULTS AND DISCUSSION

4.1 Sample collection and isolation

Ninety strains were obtained from various sources in Thailand (Table 5). They were isolated from fermented mussels (49 strains), fermented fish (25 strains), fermented pork (15 strains), bark of *Tamarindus indica*. (1 strain).

Table 5 Sample, location, strain number and number of strains

Sample	Location	Strain no.	Total
Fermented mussels (*Hoi-dong*)	Samutprakarn	LM1-1, LM1-2, LM2-1, LM2-2, LM2-3	5
	Bangkok	LM3-1, LM3-2, LM4-1, LM4-2, LM16-1, LM16-2, LM16-3, LM17-2, LM17-3, LM17-4, LM17-5, LM17-6, LM17-7, LM18-2, LM18-3, LM18-4	16
	Rayong	LM5-1, LM5-2, LM6-1, LM6-2	4
	Samutsongkhram	LM7-1SP, LM7-2S, LM7-2-2B, LM7-3, LM8-2, LMK9-1, LMK9-2L, LMK9-3	8
	Samutsakhon	LM10-1M, LM10-2M, LM10-3M, LMK11-2, LMK11-3	5
	Nakhonpathom	LM12-1, LM12-2,	4

Sample	Location	Strain no.	Total
		LM13-1, LM13-3	
	Chonburi	LM14-1, LM14-2, LM15-1P, LM15-2, LM15-2A, LM15-2B, LM15-3	7
Total strains isolated from Thai fermented mussel (*Hoi-dong*)			**49**

Table 5 Sample, location, strain number and number of strains

Sample	**Location**	**Strain no.**	**Total**
Fermented fish (*pla-paeng-daeng*)	Nakhon Si Thammarat	PD1-1, PD1-2, PD2-1, PD2-2, PD3-1, PD3-2, PD4-1, PD4-2, PD5-1, PD5-2	10
	Songkhla	PD6-1, PD6-2, PD6-3, PD7-1, PD7-2, PD8-1, PD8-2, PD9-1, PD9-2, PD10-1, PD10-2	11
	Satul	PD11-1, PD11-2, PD12-1, PD12-2	4
Total strains isolated from Thai fermented fish (*pla-paeng-daeng*)			**25**
Fermented pork (*Nham*)	Bangkok	NH1-1, NH1-2, NH1-3, NH1-4, NH1-5, NH1-6, NH1-7	7
	Pathumthani	NH2-1, NH2-2, NH2-3, NH2-4, NH2-5A, NH2-6, NH2-6A, NH2-7C	8
Total strains isolated from Thai fermented pork (*Nham*)			15
Bark of *Tamarindus indica*	Chiang Mai	BCM23-1	1
Total strain isolated from bark of *Tamarindus indica*			**1**
		Total number of strains	**90**

4.2 Identification

4.2.1 LAB from fermented mussel (*hoi-dong*)

Forty-nine strains were isolated from Thai fermented mussel (Hoi-dong) samples from various Thai provinces (Table 5). All strains were Gram-positive, catalase-negative, and facultatively anaerobic. They did not reduce nitrate. They belonged to the members of genera *Companilactobacillus*, *Lentilactobacillus*,

Lactiplantibacillus, *Lacticaseibacillus*, *Pediococcus*, *Enterococcus*, *Lactococcus* and *Leuconostoc*, and they were divided into 8 Groups when the hierarchical cluster was analyzed based on their phenotypic characteristics, and the 16S rRNA gene sequence similarity of the representative strains was determined (Figure 6 and Table 6).

Group I included ten rod-shaped strains (LM15-2A, LM16-2, LM10-2M, LM15-2B, LM18-3, LM7-1SP, LM7-2S, LM10-3M, LM10-1M and LM15-3). They did not produced gas from glucose. They grew at pH 3, in 8% NaCl, at 15°C and 45°C but did not grow at and pH 9.0. They hydrolyzed arginine. The strains contained *meso*-DAP in the cell wall. They produced D-lactic acid. All strains did not produce acid from arabinose, cellobiose, lactose, mannitol, melibiose, raffinose, rhamnose and sorbitol. The representative strains in this group showed 99.56% to 100% 16S rRNA gene sequence similarity to *Companilactobacillus formosensis* S215^{T} (Figure 6). Therefore, they were identified as *Companilactobacillus formosensis* (Zheng et al., 2020). Their differential phenotypic characteristics are presented in Table 6.

Group II included eight rod-shaped strains (LM17-6, LM17-7, LM17-2, LM17-5, LM17-4, LMK9-3, LM7-3, and LM18-4). They produced gas from glucose. They grew at pH 3 and 9, 15°C and 45 °C and in 8% NaCl. The strains did not have *meso*-DAP in the cell wall. They produced DL-lactic acid. All strains did not produce acid from lactose, mannose, rhamnose and salicin. They could hydrolyze arginine. The representative strains in this group showed 99.81% to 99.93% 16S rRNA gene sequence similarity to *Lentilactobacillus buchneri* JCM 1115T (Figure 6). Therefore, they were identified as *Lentilactobacillus buchneri* (Zheng et al., 2020). Their differential phenotypic characteristics are presented in Table 6

Group III included seventeen rod-shaped stains (LM16-1, LM6-1, LM7-2-2B, LM15-1P, LM6-2, LM14-1, LM14-2, LM15-2, LM12-1, LM18-2, LMK11-2, LM12-2, LM2-3, LM3-2, LM3-1, LM16-3, LMK11-3). They did not produce gas from glucose. They grew at pH 3 and in 8% NaCl. The strains contained *meso*-DAP in the cell wall. They produced DL-lactic acid. The representative strains in this group showed 99.78% to 100% 16S rRNA gene sequence similarity to *Lactiplantibacillus plantarum* subsp. *plantarum* ATCC 14917^{T} (Figure 6). Therefore, they were identified as *Lactiplantibacillus plantarum* subsp. *plantarum* (Zheng et al., 2020). Their differential phenotypic characteristics are presented in Table 6.

Group IV included one rod-shaped strains (LM1-1). They did not produce gas from glucose. They grew at pH 3 and, 15°C and 45 °C and in 6% and 8% NaCl but did not grow at pH 9. The strains did not have *meso*-DAP in the cell wall. They produced L-lactic acid. They did not produce acid from aesculin. It hydrolyzed arginine. The representative strain in this group showed 100% 16S rRNA gene sequence similarity to *Lacticaseibacillus rhamnosus* JCM 1136^T (Figure 6). Therefore, it was identified as *Lacticaseibacillus rhamnosus* (Zheng et al., 2020).

Group V included six tetracoccal strains (LMK9-1, LM13-1, LM17-3, LM13-3, LM5-2, and LM5-1). They did not produce gas from glucose. They grew at pH 3 and, 15°C, and in 8% NaCl but did not grow at pH 9. The strains did not have *meso*-DAP in the cell wall. They produced DL-lactic acid. The representative strains in this group included LMK9-1, LM13-1, LM17-3, LM13-3, which showed 99.86% to 100% 16S rRNA gene sequence similarity to *Pediococcus pentosaceus* DSM 20336^T (Figure 6), and isolate LM5-1 showed 99.93% 16S rRNA gene sequence similarity to *Pediococcus acidilactici* DSM 20284^T (Figure 6). Their differential phenotypic characteristics are presented in Table 6.

Group VI included four coccal strains (LM4-1, LM4-2, LM1-2 and LM2-1). They did not produce gas from glucose. They grew at pH 3 and 9, 15°C and 45°C, and in 6% and 8% NaCl. The isolates did not have *meso*-DAP in the cell wall. They produced L-lactic acid. All strains did not produce acid from arabinose. Variable acid production was found in galactose, mannitol, melibiose, raffinose, rhamnose, salicin, sorbitol, sucrose, trehalose, xylose and aesculin. They hydrolyzed arginine. The representative strain LM4-1 and LM4-2 showed 100% 16S rRNA gene sequence similarity to *Enterococcus thailandicus* DSM 21767^T (Figure 6), strain LM1-2 showed 100% 16S rRNA gene sequence similarity to *Enterococcus hirae* ATCC 9790^T (Figure 6), and LM2-1 showed 99.63% 16S rRNA gene sequence similarity (Table 6) to *Enterococcus durans* NBRC 100479^T (Figure 6).

Group VII included two coccal strains (LM2-2 and LM8-2). They did not produce gas from glucose. They grew at pH 3 and 9, 15°C, and in 6% but did not grow at 45°C, pH 9 and in 8% NaCl. They produced L-lactic acid. All strains did not produce acid from raffinose, rhamnose, sorbitol and aesculin. They hydrolyzed arginine. The representative strain LM2-2 showed 100% 16S rRNA gene sequence

similarity to *Lc. lactis* subsp. *lactis* JCM 5805^T (Figure 6), and strain LM8-2 showed 99.93% 16S rRNA gene sequence similarity to *Lc. lactis* subsp. *hordniae* NBRC 100931^T (Figure 6) and they were identified as *Lc. lactis*.

Group VIII included one coccal strain (LMK9-2L). They produced gas from glucose. They grew at pH 9, 15°C and 45°C, and in 6% but did not grow at pH 3 and in 8% NaCl. The isolates did not have *meso*-DAP in the cell wall. They produced D-lactic acid. All strains did not produce acid from cellobiose, mannitol, rhamnose, sorbitol, trehalose and aesculin. They did not hydrolyze arginine. The representative strain LMK9-2L showed 99.71% 16S rRNA gene sequence similarity to *Leuconostoc lactis* JCM 6123^T (Figure 6) and was identified as *Leuconostoc lactis*.

Table 6 Phenotypic characteristics of strains

Characteristics	I	II	III	IV	V	VI	VII	VIII
No. of strain	10	8	17	1	6	4	2	1
Cell shape	Rods	Rods	Rods	Rods	Tetracocci	Cocci in chains	Cocci in chain	Cocci in chains
Gas from glucose	-	+	-	-	-	-	-	+
Growth in 6% NaCl	+	+	+	+	+	+	+	+
Growth in 8% NaCl	+	+	+	+	+	+	-	-
Growth at pH 3	+	+	+	+	+	+	+	-
pH 9	-	+	+ (-5)	-	-	+	+	+
Growth at 15 °C	+	+	+	+	+	+	+	+
45 °C	+	+	+ (-1)	+	- (+1)	+	-	+
Arginine hydrolysis	+	+	+ (-4)	+	- (+1)	+	+	-
Acid from:								
L-Arabinose	-	+	+	+	+	-	+	+
D-Cellobiose	-	-(+1)	+	+	+	+	w1	-
Fructose	+	+	+	+	+	+	+	+
D-Galactose	+	+	+	+	+	+ (-1)	+	+
D-Glucose	+	+	+	+	+	+	+	+
D-Lactose	-	-	+	+	+ (-1)	+	+	+
D-Mannose	+	-	+	+	+	+	+	+
D-Maltose	w5	+	+	+	+ (-1)	+	+	+
D-Mannitol	-	-(+1)	+ (-1)	+	- (+1)	+ (-1)	+	-
D-Melibiose	-	+	+ (-1)	+	w3	+ (-1)	w1	+
D-Raffinose	-	w4	+ (-1)	+	w3	+ (-1)	-	+
L-Rhamnose	-	-	+ (-1)	+	- (+2)	w2	-	-
D-Ribose	+	+	+	+	+	+	+	+
Salicin	+	-	+	+	+	+ (-1)	+	+
D-Sorbitol	-	- (+1)	+ (-2)	+	- (+1)	+ (-1)	-	-
D-Sucrose	+	+ (-3)	+	+	+ (-1)	+ (-1)	w1	+
D-Trehalose	+ (-4)	+	+	+	+	+ (-1)	+	-
D-Xylose	+ (-2)	+	+	+	+	+ (-1)	+	+
Aesculin	+	+	+ (-2)	-	+	+ (-1)	-	-
meso-DAP	+	-	+	-	-	-	-	-
Isomer of lactic acid	D	DL	DL	L	DL	L	L	D

+, positive reaction; -, negative reaction; w, weakly positive. Numbers in parentheses indicate the number of strains showing the reaction

From these findings, *C. formosensis*, *L. buchneri*, *L. plantarum* subsp. *plantarum*, *L. rhamnosus*; *P. pentosaceus* and *P. acidilactici*; *En. thailandicus*, *En. hirae* and *En. durans*; *Lc. lactis* subsp. *lactis*, *Lc. lactis* subsp. *hordinae*, and *Len. lactis* strains were distributed in Thai fermented mussel (*Hoi-dong*).

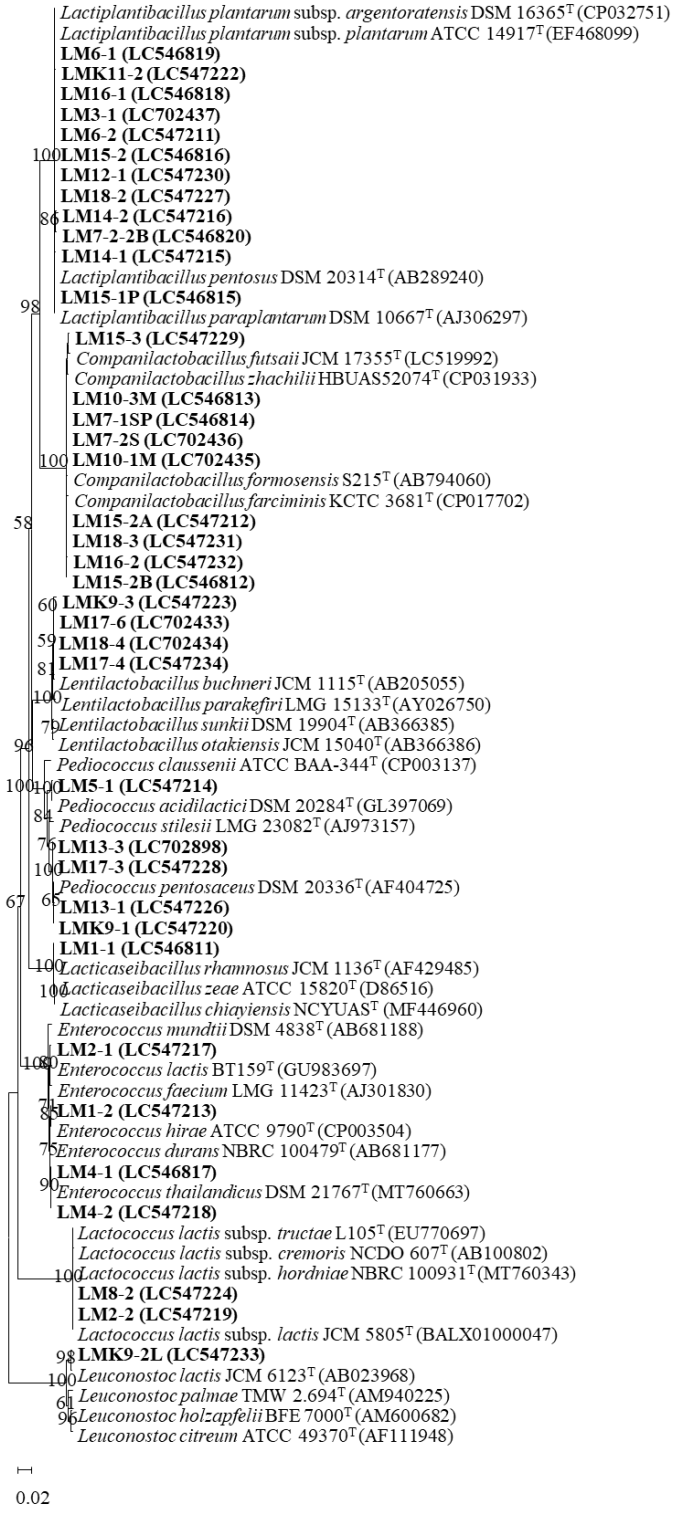


Figure 6 Neighbor-joining tree based on 16S rRNA gene of the representative strains from *hoi-dong*

4.2.2 LAB from fermented fish (*pla-paeng-daeng*)

Twenty-five strains were isolated from Thai fermented fish (*pla-paeng-daeng*) samples from various Thai provinces (Table 5). All strains were Gram-positive, catalase-negative, and facultatively anaerobic. They did not reduce nitrate. They were belonged to the members of genera *Lactiplantibacillus*, *Limosilactobacillus*, *Companilactobacillus*, and *Enterococcus*, and they were divided into 7 Groups when the hierarchical cluster was analyzed based on their phenotypic characteristics, and the 16S rRNA gene sequence similarity of the representative isolates was determined (Figure 7 and Table 7).

Group I included six rod-shaped strains (PD3-1, PD6-2, PD11-1, PD8-1, PD9-2 and PD6-1). They did not produce gas from glucose. They grew at pH 3 and 9, in 6% and 8% NaCl, at 15°C but did not grow at 45°C. They did not hydrolyze arginine. The strains contained *meso*-DAP in the cell wall. They produced DL-lactic acid. Variable acid production was found in arabinose, galactose, melibiose, raffinose, and ribose. The representative strains in this group showed 99.71% to 100% 16S rRNA gene sequence similarity to *Lactiplantibacillus pentosus* DSM 20314^{T} (Figure 7). Therefore, they were identified as *Lactiplantibacillus pentosus*; their differential phenotypic characteristics are presented in Table 7.

Group II included one rod-shaped strain (PD9-1). It did not produce gas from glucose. It grew at pH 3 and 9, 15 °C and 45 °C, and in 6% and 8% NaCl. It contained *meso*-DAP in the cell wall. It produced DL-lactic acid. It did not produce acid from aesculin. It could not hydrolyze arginine. The representative strain in this group showed 99.85% 16S rRNA gene sequence similarity to *Lactiplantibacillus argentoratensis* DSM 16365^{T} (Figure 7) (Zheng et al., 2020). Therefore, it was identified as *Lactiplantibacillus argentoratensis*; its phenotypic characteristics are presented in Table 7.

Group III included two rod-shaped strains (PD10-1 and PD8-2). They produced gas from glucose. They grew at pH 3 and in 8% NaCl but did not grow at pH 9. The strains did not contain *meso*-DAP in the cell wall. They produced DL-lactic acid. They could hydrolyze arginine. All strains did not produce acid from cellobiose, galactose, lactose, mannose, mannitol, raffinose, rhamnose, salicin, sorbitol, trehalose, and aesculin. The representative strains in this group showed 99.49% 16S rRNA gene

sequence similarity to *Limosilactobacillus fermentum* CECT 562^T (Figure 7) (Zheng et al., 2020). Therefore, they were identified as *Limosilactobacillus fermentum*; their differential phenotypic characteristics are presented in Table 7.

Group IV included four rod-shaped strains (PD12-2, PD7-1, PD6-3 and PD4-2). They did not produce gas from glucose. They grew at pH 3 and pH 9, 15°C and 45 °C and in 6% and 8% NaCl. The strains did not have *meso*-DAP in the cell wall. They produced DL-lactic acid. They did not produce acid from cellobiose, lactose, maltose, mannitol, melibiose, rhamnose, ribose, and trehalose. Variable acid production was found in xylose and aesculin. They hydrolyzed arginine. The representative strains in this group showed 99.63% to 99.71% 16S rRNA gene sequence to *Companilactobacillus pabuli* NFFJ11^T (Figure 7) (Zheng et al., 2020). Therefore, they were identified as *Companilactobacillus pabuli*; their differential phenotypic characteristics are presented in Table 7.

Group V included five rod-shaped strains (PD11-2, PD12-1, PD5-2, PD7-2, and PD10-2). They did not produce gas from glucose. They grew at pH 3 and pH 9, 15°C, and 45 °C and in 6% and 8% NaCl. They hydrolyzed arginine. The strains did not have *meso*-DAP in the cell wall. They produced L-lactic acid. They did not produce acid from arabinose, cellobiose, lactose, mannitol, melibiose, raffinose, rhamnose, ribose, sorbitol, trehalose, and xylose. Variable acid production was found in maltose, salicin, sucrose and aesculin. The representative strains in this group showed 99.77% to 99.85% 16S rRNA gene sequence similarity to *Companilactobacillus farciminis* KCTC 3681^T (Figure 7). Therefore, they were identified as *Companilactobacillus farciminis*; their differential phenotypic characteristics are presented in Table 7.

Group VI included six rod-shaped strains (PD5-1, PD4-1, PD1-1, PD1-2, PD2-2 and PD2-1). They did not produce gas from glucose. They grew at pH 3 and 9, 15°C and 45°C, and in 6% and 8% NaCl. They variably hydrolyzed arginine. The strains did not have *meso*-DAP in the cell wall. They produced L-lactic acid. All strains did not produce acid from cellobiose, maltose, mannitol, melibiose, raffinose, rhamnose, ribose, sorbitol, trehalose, and xylose. Variable acid production was found in arabinose, galactose, lactose and aesculin. The representative strains showed 100% 16S rRNA gene sequence similarity to *Companilactobacillus futsaii* JCM 17355^T

(Figure 7). Therefore, they were identified as *Companilactobacillus futsaii*; their differential phenotypic characteristics are presented in Table 7.

Group VII included one coccal strain (PD3-2). It did not produce gas from glucose. They grew at pH 3 and 9, 15°C and 45°C, and in 6% and 8% NaCl. It hydrolyzed arginine. The strain did not have *meso*-DAP in the cell wall. It produced L-lactic acid. The isolate did not produce acid from raffinose, rhamnose, sorbitol, sucrose, and xylose. The representative strain PD3-2 showed 99.54% 16S rRNA gene sequence similarity to *Enterococcus lactis* BT159^{T} (Figure 7). Therefore, it was identified as *Enterococcus lactis*; its differential phenotypic characteristics are presented in Table 7.

Table 7 Phenotypic characteristics of strains

Characteristics	I	II	III	IV	V	VI	VII
No. of strain	6	1	2	4	5	6	1
Cell shape	Rods	Rods	Rods	Rods	Rods	Rods	Cocci in chains
Gas from glucose	-	-	+	-	-	-	-
Growth in 6% NaCl	+	+	+	+	+	+	+
Growth in 8% NaCl	+	+	+	+	+	+	+
Growth at pH 3	+	+	+	+	+	+	+
pH 9	+	+	-	+	+	+	+
Growth at 15 °C	+	+	+	+	+	+	+
45 °C	-	+	+	+	+	+	+
Arginine hydrolysis	-	-	+	+	+	+(-1)	-
Acid from:							
L-Arabinose	+(-2)	+	+	+	-	-(+2)	+
D-Cellobiose	+	+	-	-	-	-	+
Fructose	+	+	+	+	+	+	+
D-Galactose	+(-2)	+	-	+	+	-(+2)	+
D-Glucose	+	+	+	+	+	+	+
D-Lactose	+	+	-	-	-	-(+1)	+
D-Mannose	+	+	-	+	+	+	+
D-Maltose	+	+	+	-	-(+2)	-	+
D-Mannitol	+	+	-	-	-	-	+
D-Melibiose	+(-2)	+	+	-	-	-	+
D-Raffinose	+(-3)	+	-	+	-	-	-
L-Rhamnose	+	+	-	-	-	-	-
D-Ribose	+(-1)	+	+	-	-	-	+
Salicin	+	+	-	+	+(-2)	+	+
D-Sorbitol	+	+	-	+	-	-	-
D-Sucrose	+	+	+	+	-(+1)	+	-
D-Trehalose	+	+	-	-	-	-	+
D-Xylose	+	+	+	+(-2)	-	-	-
Aesculin	+	-	-	+(-2)	-(+1)	-(+1)	+
meso-DAP	+	+	-	-	-	-	-
Isomer of lactic acid	DL	DL	DL	DL	L	L	L

+, positive reaction; -, negative reaction. Numbers in parentheses indicate the number of strains showing the reaction

In Thai fermented fish (*pla-paeng-daeng*), this result provided that the distribution of LAB mainly was belong to the *C. futsaii*, *C. farciminis*, *C. pabuli*, *L. pentosus*, *L. argentoratensis*, *L. fermentum*, and *En. lactis*.

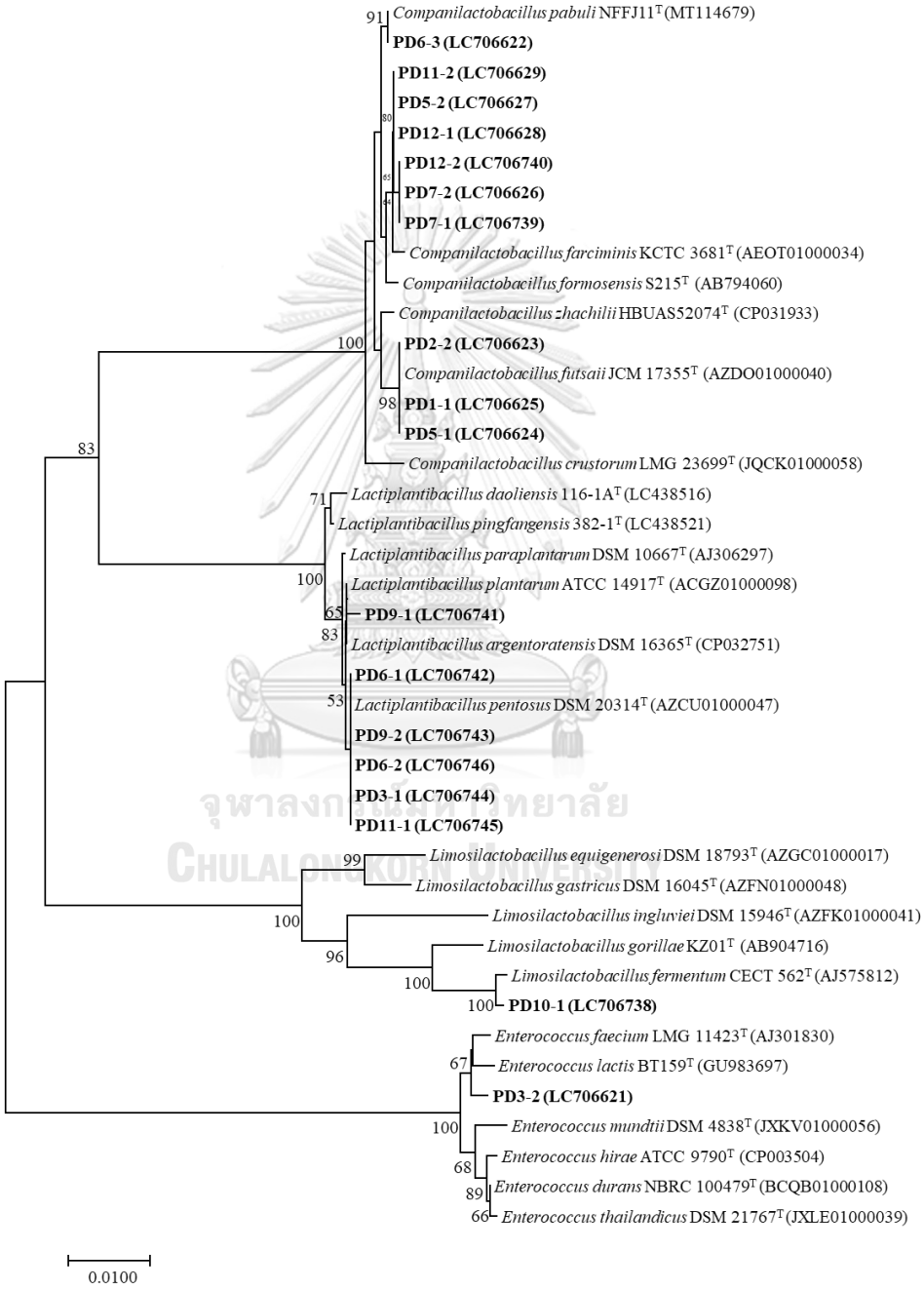


Figure 7 Neighbor-joining tree based on 16S rRNA gene of the representative strains from *pla-paeng-daeng*

4.2.3 LAB from fermented pork (*nham*)

Fifteen strains were isolated from Thai fermented pork (*Nham*) samples from various Thai provinces (Table 5). All strains were Gram-positive, catalase-negative, and facultatively anaerobic. They did not reduce nitrate. They belonged to the members of genera *Lactiplantibacillus, Lacticaseibacillus, Pediococcus,* and *Lactococcus,* they were divided into 5 Groups when the hierarchical cluster was analyzed based on their phenotypic characteristics, and the 16S rRNA gene sequence similarity of the representative strains were determined (Figure 8, and Table 8).

Group I included four rod strains (NH1-2, NH1-5, NH2-1, and NH2-2). They did not produce gas from glucose. They grew at pH 3 and 9, 15°C, and in 6 % and 8% NaCl but did not grow at 45°C. They could not hydrolyze arginine, but variably produce acid from rhamnose and aesculin. The strains contained *meso*-DAP in the cell wall. They produced DL-lactic acid. The representative strains in this group included showed 99.71% to 100% 16S rRNA gene sequence similarity to *Lactiplantibacillus pentosus* DSM 20314^{T} (Zheng et al., 2020) (Figure 8). Their phenotypic characteristics are presented in Table 8. Thus, they were identified as *L. pentosus*.

Group II included two rod strains (NH1-1 and NH2-4). They did not produce gas from glucose. They grew at pH 3 and 9, 15°C and 45°C, and in 6 % and 8% NaCl. They could not hydrolyze arginine. The strains contained *meso*-DAP in the cell wall. They produced DL-lactic acid. The representative strains in this group included showed 99.93% to 100% 16S rRNA gene sequence similarity to *Lactiplantibacillus argentoratensis* DSM 16365^{T} (Zheng et al., 2020) (Figure 8). Their phenotypic characteristics are presented in Table 8. Thus, they were identified as *L. argentoratensis*.

Group III contained one rod strain (NH2-5A). They did not produce gas from glucose. They grew at pH 3 and 9, 15°C and 45°C, and in 6 % and 8% NaCl. It did not hydrolyze arginine and did not produce acid from aesculin. The strains did not have *meso*-DAP in the cell wall. They produced L-lactic acid. The representative strain in this group included showed 99.79% 16S rRNA gene sequence similarity to *Lacticaseibacillus paracasei* subsp. *tolerans* JCM 1171^{T} (Zheng et al., 2020) (Figure 8). Its phenotypic characteristics are presented in Table 8. Thus, it was identified as *L. paracasei* subsp. *tolerans.*

Group IV included seven tetracoccal strains (NH1-3, NH1-4, NH1-6, NH1-7, NH2-3, NH2-6 and NH2-6A). They did not produce gas from glucose. They grew at pH 3 and 9, 15°C, and in 6 % and 8% NaCl but did not grow at 45°C. They could variably hydrolyze arginine. The strains did not have *meso*-DAP in the cell wall. They produced DL-lactic acid. The representative strains in this group included showed 99.51% to 100% 16S rRNA gene sequence similarity to *Pediococcus pentosaceus* DSM 20336^T (Figure 8). Their differential phenotypic characteristics are presented in Table 8. Thus, they were identified as *P. pentosaceus*.

Table 8 Phenotypic characteristics of strains

Characteristics	I	II	III	IV	V
No. of strain	4	2	1	7	1
Cell shape	Rods	Rods	Rods	Tetradcocci	cocci
Gas from glucose	-	-	-	-	-
Growth in 6% NaCl	+	+	+	+	+
Growth in 8% NaCl	+	+	+	+	+
Growth at pH 3	+	+	+	+	+
pH 9	+	+	+	+	+
Growth at 15 °C	+	+	+	+	+
45 °C	-	+	+	+	-
Arginine hydrolysis	-	-	-	+(-2)	+
Acid from:					
L-Arabinose	+	+	+	+	+
D-Cellobiose	+	+	+	+	+
Fructose	+	+	+	+	+
D-Galactose	+	+	+	+	+
D-Glucose	+	+	+	+	+
D-Lactose	+	+	+	+	+
D-Mannose	+	+	+	+	+
D-Maltose	+	+	+	+	+
D-Mannitol	+	+	+	-	+
D-Melibiose	+	+	+	- (+1)	-
D-Raffinose	+	+	+	-(+1)	-
L-Rhamnose	+(-1)	+	+	+(-2)	-
D-Ribose	+	+	+	+	+
Salicin	+	+	+	+	+
D-Sorbitol	+	+	+	-(+1)	-
D-Sucrose	+	+	+	+(-1)	+
D-Trehalose	+	+	+	+	+
D-Xylose	+	+	+	+	+
Aesculin	+(-1)	+	-	-(+3)	+
meso-DAP	+	+	-	-	-
Isomer of lactic acid	DL	DL	L	DL	L

+, positive reaction; -, negative reaction.. Numbers in parentheses indicate the number of strains showing the reaction

Group V included one coccal strain (NH2-7C). They did not produce gas from glucose. They grew at pH 3, 15°C, and in 6% and 8% NaCl but did not grow at 45°C, and pH 9. They produced L-lactic acid. The strain did not produce acid from melibiose, raffinose, rhamnose, and sorbitol. They hydrolyzed arginine. The representative strain NH2-7C showed 99.93% 16S rRNA gene sequence similarity to *Lc. lactis* subsp. *lactis* JCM 5805^T (Figure 8). Thus, it was identified as *Lc. lactis* subsp. *lactis*.

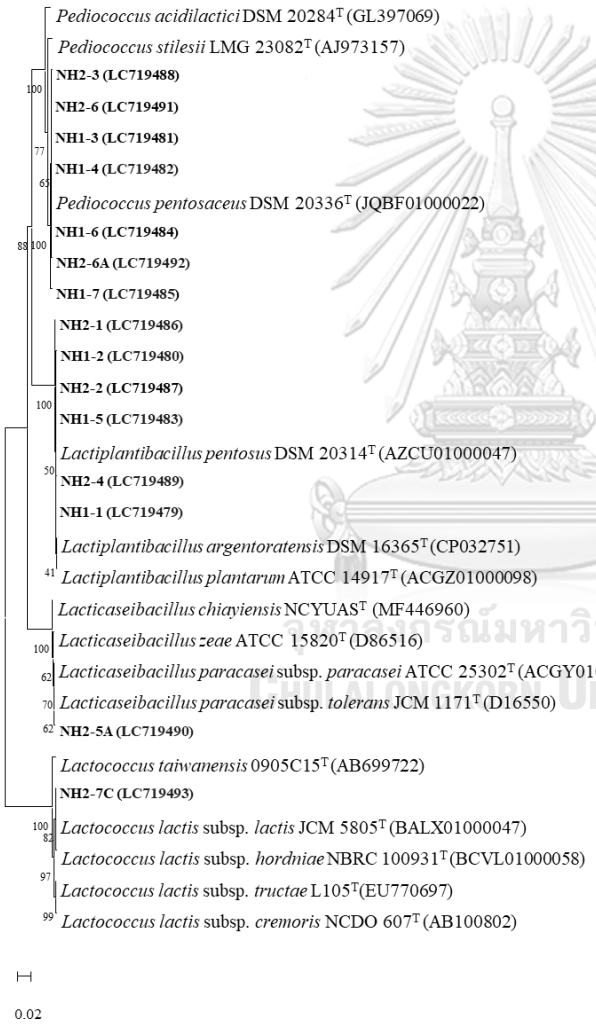


Figure 8 Neighbor-joining tree based on 16S rRNA gene of the representative strains from *nham*

In Thai fermented pork (nham), LAB mainly was belonged to L. pentosus, L. argentoratensis, L. paracasei subsp. tolerans, P. pentosaceus, and Lc. lactis subsp. lactis.

4.2.4 LAB from tree bark of *Tamarindus indica*

Description of *Terrilactibacillus tamarindi* sp. nov.

Terrilactibacillus tamarindi (ta.ma.rin'di., N.L. gen. n. *tamarindi* of *Tamarindus indica* which the type strain was isolated)**.** Cells of strain BCM23-1^T were Gram-stain-positive, catalase-positive, facultatively anaerobic, endospore-forming rods (0.5–0.9×4.3–5.8μm) as shown in Figure 9 (A). Colonies on MRS agar plates were circular, convex and white (1.2–1.3 mm in diameter). D-Lactic acid was produced from glucose. Peritrichous flagella were observed as shown in Figure 9 (B). The strain showed negative results for oxidase activity, hydrolysis of arginine, casein, gelatin and starch, and nitrate reduction. Growth was observed at 20–45 °C (optimum, 30 °C), at pH from pH 3.5 to 9.0 (optimum, pH 7.0) and with 1–4% (w/v) NaCl, but no growth was observed at 10 °C. In API 50 CH tests, acid is positive for D-glucose, D-fructose, inulin, maltose, D-mannose, sucrose, trehalose, raffinose, turanose, potassium gluconate (weak), potassium 2-ketogluconate (weak) and potassium 5-ketogluconate (weak), but negative for glycerol, erythritol, D-galactose, D-arabinose, L-arabinose, D-ribose, D-xylose, L-xylose, D-adonitol, methyl-β-D-xylopyranoside, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, methyl-α-D-mannopyranoside, methyl-α-D-glucopyranoside, N-acetylglucosamine, amygdalin, arbutin, aesculin, salicin, cellobiose, lactose, melibiose, melezitose, starch, glycogen, xylitol, gentiobiose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol and L-arabitol. Additionally, in API ZYM assays, positive for acid phosphatase and naphthol-AS-BI-phosphohydrolase but negative for alkaline phosphatase, β-galactosidase, β-glucosidase, N-acetyl-βglucosaminidase, esterase lipase (C8), lipase (C14), valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, β-glucuronidase, α-mannosidase and α-fucosidase. Weak enzymatic activities are found for esterase (C4), leucine arylamidase, α-galactosidase and α-glucosidase. The different phenotypic characteristics are described in Table 9.

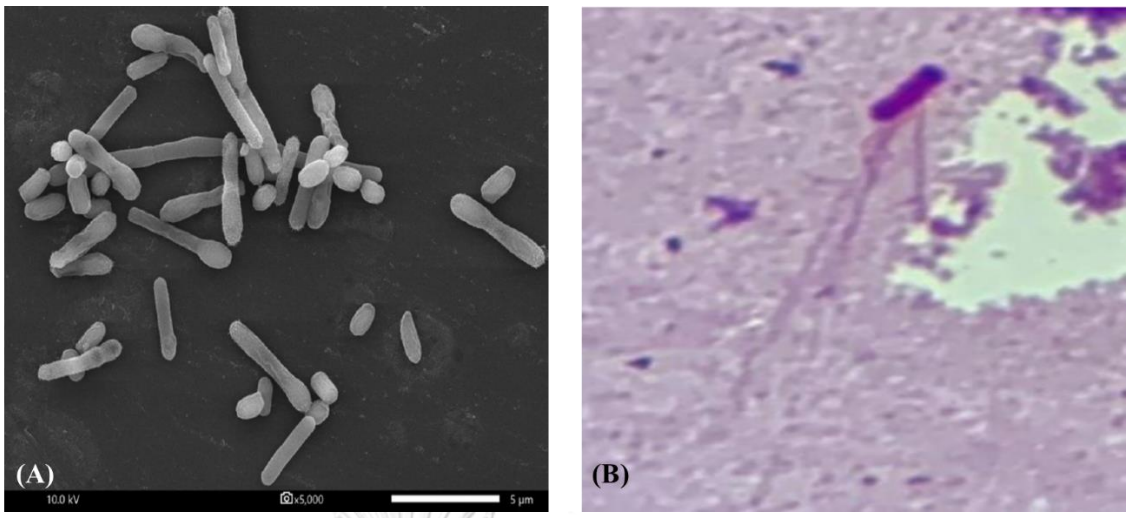


Figure 9 Scanning electron micrograph of strain BCM23-1^T grown on GYP agar at 30°C for 7 days (A) and Photomicrograph of terminal endospores (A) and flagella of strain BCM23-1 (B)

Table 9 Differential characteristics of strain BCM23-1^T and its related type strain

Strains: 1, BCM23-1^T; 2, *Terrilactibacillus laevilacticus* NK26-11^T. All data were obtained in this study. +, Positive; w, weakly positive; −, negative.

Characteristics	**1**	**2**
Maximum NaCl for growth (%)	4	3
pH range for growth	3.5-9.0	3.5-8.0
Acid production from:		
N-Acetylglucosamine	-	+
D-Galactose	-	w
Inulin	+	-
D-Lyxose	-	w
Melezitose	-	w
Melibiose	-	w
Methyl-α-D-glucopyranoside	-	+
Potassium-5-ketogluconate	w	-

Table 9 Differential characteristics of strain BCM23-1^T and its related type strain

Strains: 1, BCM23-1^T; 2, *Terrilactibacillus laevilacticus* NK26-11^T. All data were obtained in this study. +, Positive; w, weakly positive; −, negative.

Characteristics	**1**	**2**
Enzyme activity:		
Acid phosphatase	+	w
Esterase (C4)	w	-
α-Galactose	w	-
α-Glucosidase	w	-
Napthol-AS-BI-phosphohydrolase	+	w

The cell-wall peptidoglycan was the directly crosslinked *meso*-diaminopimelic acid type A1γ as reported in the family *Bacillaceae* (Schleifer & Kandler, 1972) and *Sporolactobacillaceae* (Chang & Stackebrandt, 2014; Schumann, 2011). MK-7 was the major menaquinone (100%), which was in accordance with *T. laevilacticus* NK26-11^T (Prasirtsak et al., 2016). Strain BCM23-1^T contained major fatty acids (>10% of the total fatty acids) $C_{18:1}\omega 9c$ (25.6%), anteiso-$C_{17:0}$ (23.6%) and anteiso-$C_{15:0}$ (22.6%) when cultivated in MRS broth and anteiso-$C_{17:0}$ (48.7%), anteiso-$C_{15:0}$ (24.5%) and iso-$C_{16:0}$ (13.2%) when cultivated on GYP agar plate. Nonetheless, there were some differences between strains BCM23-1^T and NK26-11^T in the amounts of $C_{16:0}$, iso-$C_{16:0}$ and anteiso-$C_{15:0}$, as shown in Table 10. Predominant polar lipids were diphosphatidylglycerol and phosphatidylglycerol, with minor amounts of an unidentified aminophospholipid, an unidentified phospholipid and an unidentified lipid (Figure 10).

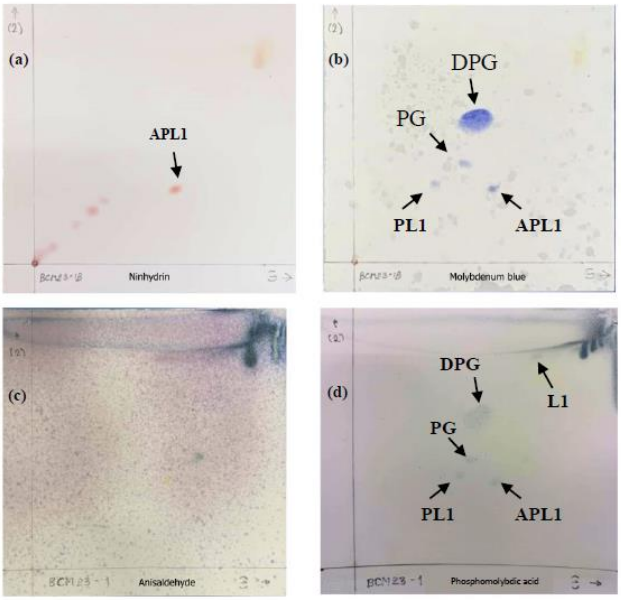


Figure 10 Polar lipid profile of strain BCM23-1^{T} on a two dimensional thin-layer chromatogram

Polar lipids were detected with ninhydrin (a), molybdenum blue (b), anisaldehyde (c) andphosphomolybdic acid (d).

Abbreviation: DPG, diphosphatidylglycerol; PG, phosphatidglycerol; AL1, unidentified aminophospholipid; PL1, unidentified phospholipid; L1, unidentified lipid.

Table 10 Cellular fatty acid composition of strain BCM23-1^T and its closely related type strain

Strains: 1, BCM23-1^T; 2, *Terrilactibacillus laevilacticus* NK26-11^T. Values are percentages of total cellular fatty acids. –, Not present. Fatty acids amounting to less than 0.5 % in all strains were omitted. Major components are indicated in bold type. All data were determined in this study.

Strain	1		2	
Growth medium	**MRS**	**GYP**	**MRS**	**GYP**
Straight-chain fatty acids:				
$C_{12:0}$	-	-	-	1.4
$C_{14:0}$	0.5	1.0	0.7	2.54
$C_{16:0}$	9.0	2.9	14.0	7.0
$C_{18:0}$	1.5	1.7	1.6	2.0
Unsaturated fatty acids:				
$C_{15:1}\omega5c$	-	0.7	-	-
iso-$C_{17:1}\omega5c$	-	0.7	-	-
$C_{18:1}\omega9c$	25.6	1.0	25.0	1.4
Branched fatty acids:				
iso-$C_{14:0}$	0.6	0.5	0.7	-
iso-$C_{15:0}$	1.5	1.4	1.5	0.8
iso-$C_{16:0}$	6.9	13.2	17.1	21.2
iso-$C_{17:0}$	0.8	1.8	0.8	1.7
iso-$C_{18:0}$	-	-	-	0.8
iso-$C_{19:0}$	1.0	-	0.9	-
$C_{17:0}$2OH	1.5	-	1.7	-
anteiso-$C_{15:0}$	22.6	24.5	10.7	9.7
anteiso-$C_{17:0}$	23.6	48.7	19.8	50.0
anteiso-$C_{19:0}$	-	0.5	-	-
Summed feature 3*	0.6	-	0.7	-
Summed feature 8†	2.8	-	2.8	-

*Summed feature 3 consisted of $C_{16:1}$ $\omega7c$ and/or $C_{16:1}\omega6c$

†Summed feature 8 consisted of $C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$.

Based on 16s rRNA gene sequence of strain BCM23-1^T (1,436 bp), the strain BCM23-1^T was closely related to *Terrilactibacillus laevilacticus* NK26-11^T (98.31 %). The phylogenetic analysis based on the neighbour-joining (NJ) algorithm demonstrated that strain BCM23-1^T formed a cluster with the genus *Terrilactibacillus* (Figure 11) based on its 16S rRNA gene sequence (1,436 bp). In addition, the topologies of phylogenetic trees built using the ML and MP methods also supported and formed a stable clade. Strain BCM23-1^T was located in a phylogenetic clade together with recognized species and was most closely related to *T. laevilacticus* NK26-11^T with 98.31% similarity.

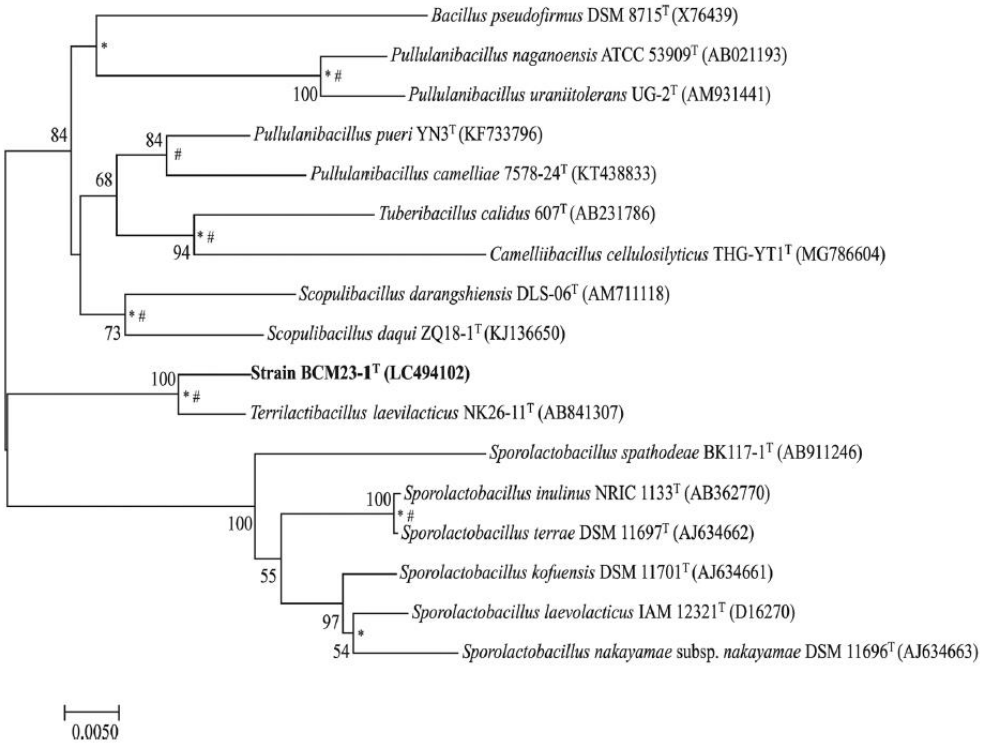


Figure 11 Neighbor-joining tree based on almost-complete 16S rRNA gene sequences showing relationships between strain BCM23-1^T and related species of the genus Terrilactibacillus. Asterisks and sharps (*,$^\#$) indicate that the corresponding nodes was also recovered in maximum-likelihood and maximum-parsimony trees, respectively.

The genome sequence of strain BCM23-1^T (WNHB00000000) obtained from this study was 3,249,523bp in size and a genome sequence of *T. laevilacticus* NK26-11^T (VDCY00000000) obtained from GenBank was 3,366,908 bp in size. The

average *in silico* DNA G+C contents of BCM23-1^T and *T. laevilacticus* NK26-11^T are 37.1 and 36.8 mol%, respectively. The genomic features of strain BCM23-1^T and *T. laevilacticus* NK26-11^T are presented in Table 11. The ANIb and ANIm values of the draft genomes between strain BCM23-1^T and the closest type strain as *T. laevilacticus* NK26-11^T were 89.9 and 90.8%, respectively which are clearly lower than the thresholds of 95–96% for species delineation. The dDDH value of the draft genome between strain BCM23-1^T and the closest type strain, *T. laevilacticus* NK26-11^T, was 40.4% (C.I. 37.9–42.9%), which is lower than the cut-off value of 70% for species delineation, therefore supporting the proposal of strain BCM23-1^T representing a new species within the genus *Terrilactibacillus* (Chun et al., 2018; Goris et al., 2007; Richter & Rosselló-Móra, 2009; Stackebrandt, 2006). A total of 3088 coding genes from the draft genome of strain BCM23-1^T were predicted by RAST. Circular genomic and the subsystem were constructed and shown in Figure 12.

Table 11 Genomic statistics of strain BCM23-1^T and *Terrilactibacillus laevilacticus* NK26-11^T

Strains: 1, BCM23-1^T; 2, *Terrilactibacillus laevilacticus* NK26-11^T. In this study, genomic statistics of strain BCM23-1^T and *T. laevilacticus* NK26-11^T were analyzed by using RAST (www.rast.theseed.org).

Attribute	1	2*
Accession no.	WNHB00000000	VDCY00000000
Genome size (bp)	3,249,543	3,366,908
G+C content (mol%)	37.1	36.8
Genome coverage	180x	80.2x
N50	112,416	294,859
Number of contigs	70	38
No. of coding sequences	3,088	3,183
No. of RNAs	68	69

*Data obtained from GenBank.

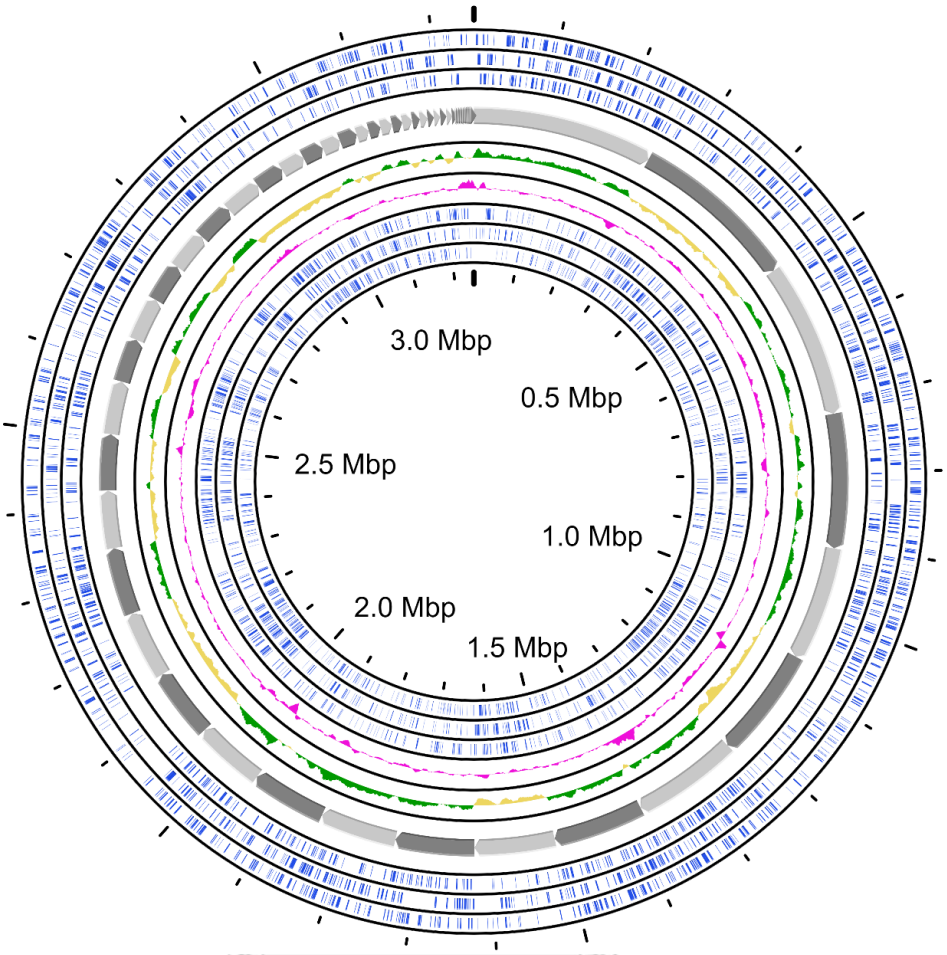


Figure 12 Circular genomic map of strain BCM23-1^{T}

The information is indicated as follows: open reading frames (ORFs) (blue), GC skew (+) (green), GC skew (-) (yellow), and GC content (pink)

On the basis of phenotypic, chemotaxonomic and genotypic characteristics, as well as draft genomic data, it is proposed that strain BCM23-1^{T} should be classified as a novel species in the genus *Terrilactibacillus*, for which the name *Terrilactibacillus tamarindi* sp. nov. is proposed. The type strain is BCM23-1^{T} (=LMG 31662^{T}=JCM 33748^{T}=TISTR 2841^{T}). The accession numbers for the 16S rRNA gene and draft genome sequences of the type strain are LC494102 and WNHB00000000, respectively.

4.3 Bile salt hydrolase activity

Bile salt hydrolase activity (BSH) activity has been considered a key associated with cholesterol-lowering activity and is also recognized as an additional criterion for the selecting probiotics (Miremadi et al., 2014). By deconjugating bile salts, BSH activity enhances bacterial growth and colonization in the gut (Máire Begley et al., 2006). Only 12 strains, eight strains of *L. plantarum* (LM6-1, LM6-2, LM7-2-2B, LM12-1, LM14-1, LM14-2, LM15-1P, and LM15-2), two strains of *L. pentosus* (PD3-1 and PD9-2), and one strain of *Lc. lactis* subsp. *lactis* NH2-7C demonstrated bile salt hydrolase activity by the formation of opaque white colonies. In addition, one strain of *En. lactis* PD3-2 demonstrated bile salt hydrolase activity by forming halos around colonies. The bile salt hydrolase activity is shown in Figure 13. Based on these findings, the formation of opaque white colonies or bile acid precipitates around the colonies were considered BSH activity (Dashkevicz & Feighner, 1989; Jayashree et al., 2014).

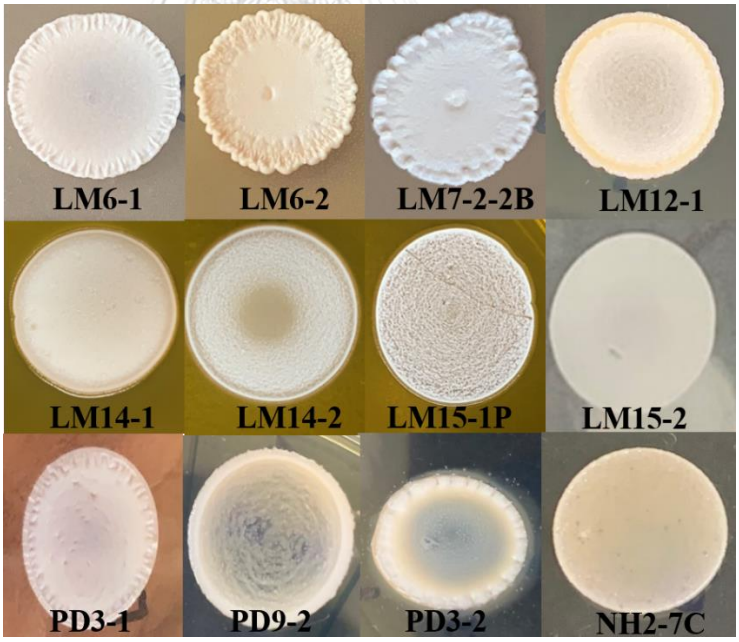


Figure 13 Bile salt hydrolase activity of selected strains on MRS agar supplemented with 0.5% taurodeoxycholic acid sodium salt (TDCA)

Based on RAST annotation database, the *bsh* gene encoding choloylglycine hydrolase was found in the LM14-2 and NH2-7C genome. The presence of BSH activity of strain and *Lc. lactis* subsp. *lactis* NH2-7C and *L. plantarum* LM14-2 was also supported by the *bsh* gene in the genomic annotation analysis (Table 19 and 25, and Figure 14). The BSH activity in LAB probiotic lowers serum cholesterol levels and makes them more resistant to bile salts (Noriega et al., 2006). Based on the screening, this research is consistent with several previous studies (Abushelaibi et al., 2017; Y. F. Liu et al., 2017). Furthermore, these findings might demonstrate that BSH-producing strains can also be found in non-human sources.

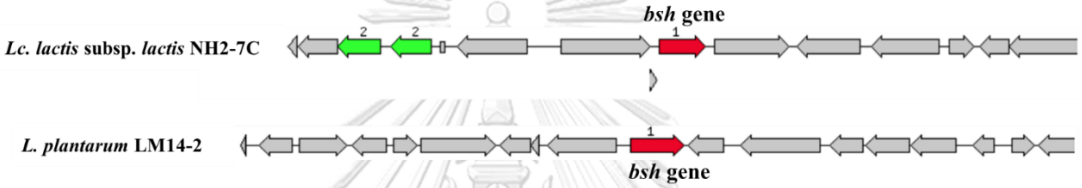


Figure 14 Determination BSH gene of strain NH2-7C and LM14-2 from RAST sever web-based tool

In the Guidelines for the Evaluation of Probiotics in Food issued by FAO/WHO, bile salt hydrolase activity relates to several desirable properties of probiotics, such as enhancing the gastrointestinal tolerance and adherence (Máire Begley et al., 2006; Joint, 2002). Furthermore, BSH activity has been recognized as an essential factor related to cholesterol-lowering effects (lower total and low-density lipoprotein (LDL cholesterol) (Jones et al., 2013), and it is also recognized as an additional criterion for the selection of probiotics and safety assurance (Miremadi et al., 2014). Conversely, the report of Máire Begley et al. (2006) reported that a high level of deconjugated bile may impair lipid digestion, disrupt normal intestinal conditions, induce gallstone formation, and may be further modified to carcinogenic secondary bile salts. After considering all the advantages and risks using the scientific evidence revealed above, this study suggested that the bile salt deconjugation property could be seen as desirable when the isolate could not modify the deconjugated bile into the harmful secondary bile products. Aside from the choloylglycine hydrolase, no genes associated with the secondary bile salts biosynthesis were discovered in strain LM14-2 and NH2-7C. This concludes the strain's bile salt deconjugation ability,

which could play vital role in the host digestive system. With regards to its inability to produce the harmful secondary bile products, this study considers LM14-2 and NH2-7C poses no safety issues from this property**.**

4.4 Cholesterol assimilation

Hypercholesterolemia is a risk factor for cardiovascular disease (CVD) that is the primary cause of mortality (Darwin R. Labarthe & Sandra B. Dunbar, 2012). Hence, reducing serum cholesterol levels is vital to preventing the disease. In this study, the selected strains exhibited that the percentage of cholesterol assimilation varied from 27.40% to 86.07% (Figure 15).

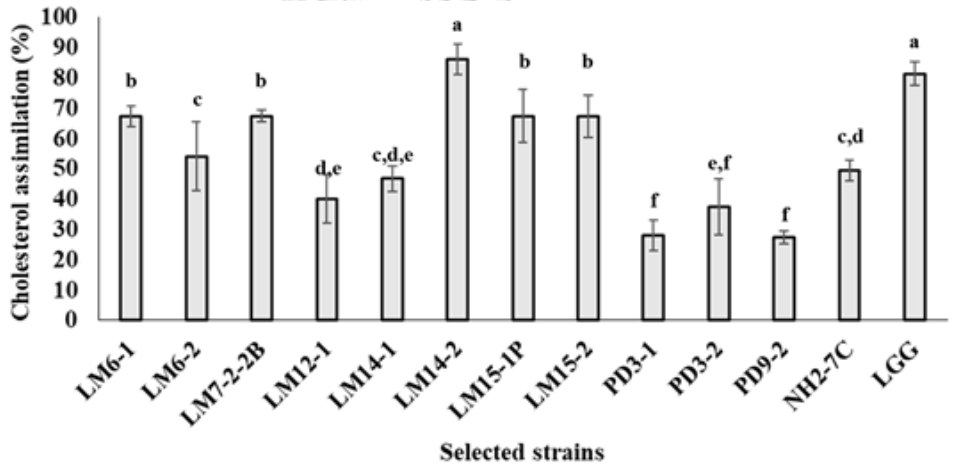


Figure 15 The cholesterol assimilation ability of selected strains

Data represent the mean ± SD. The different alphabets mean significant difference ($P \leq 0.05$)

L. plantarum LM14-2 (86.07 ± 5.03%) showed the highest cholesterol assimilation rate, followed by *L. plantarum* LM7-2-2B (67.40 ± 2.00%), *L. plantarum* LM6-1 (67.40 ± 3.46%), *L. plantarum* LM15-2 (67.40 ± 6.93%), and *L. plantarum* LM15-1P (67.40 ± 8.72%), *L. plantarum* LM6-2 (54.07 ± 11.31%), *Lc. lactis* subsp. *lactis* NH2-7C (49.40 ± 3.46%), *L. plantarum* LM14-1 (46.73 ± 4.16%), *L. plantarum* LM12-1 (40.07 ± 8.08%), *En. lactis* PD3-2 (37.40 ± 9.17%), *L. pentosus* PD3-1 (28.07 ± 5.03%), and *L. pentosus* PD9-2 (27.40 ± 2.00%). Remarkably, *L. plantarum* LM14-2 showed the potential cholesterol ability similar as *L. rhamnosus* GG (81.40 ± 4.00%). Hence, it could be concluded that the amount of cholesterol assimilated revealed a wide variation among strains. The capacity of cholesterol

assimilation observed in this study is consistent with the findings of various investigations (Miremadi et al., 2014; Shehata et al., 2016; Tomaro-Duchesneau et al., 2014). BSH and cholesterol assimilation activities are the cholesterol removal mechanisms and desirable characteristics of probiotics (Ishimwe et al., 2015). Probiotics can consume cholesterol for their metabolism (Bordoni et al., 2013). As a result, luminal cholesterol levels available for absorption are reduced.

4.5 Gastrointestinal transit tolerance

When probiotics are administered, they transit into the gastrointestinal tract (Han et al., 2021). To function as probiotics in the gastrointestinal system and perform their beneficial effect on the host, the microorganisms must be able to withstand the gastrointestinal condition. Therefore, the selected strains were determined the viability during gastrointestinal transit. The survival of selected strains after exposure to the simulated gastric condition and simulated small intestinal condition is shown in Table 12. The reduction of viable cells was observed through the incubation period in simulated gastric condition. The survival of selected strains was decreased by about 0.5-3.2 log cycle after incubation in simulated gastric condition at pH 3.0 for 3 h. In simulated gastric condition, the viability of *Lc. lactis* subsp. *lactis* NH2-7C was slightly decreased from 8.56 ± 0.07 to 8.05 ± 0.14 (log_{10}CFU/ml); while, the viability of *L. rhamnosus* GG was reduced from 9.38 ± 0.04 to 7.78 ± 0.18 (log_{10}CFU/ml) (Table 12).

Table 12 The viability during gastrointestinal transit of selected strains

Selected strains	Simulated gastric condition (log_{10}CFU/ml)[a]		Simulated intestinal condition (log_{10}CFU/ml)[a]	
	0 h	**3 h**	**0 h**	**5 h**
L. plantarum LM6-1	8.93 ± 0.08	6.52 ± 0.07*,**	5.95 ± 0.23	4.60 ± 0.21*
L. plantarum LM6-2	9.12 ± 0.10	7.66 ± 0.22*	5.93 ± 0.29	4.08 ± 0.04*,**
L. plantarum LM7-2-2B	9.41 ± 0.05	8.18 ± 0.13*	6.79 ± 0.10	4.55 ± 0.13*
L. plantarum LM12-1	9.16 ± 0.09	7.52 ± 0.07*	7.17 ± 0.07	4.17 ± 0.07*,**
L. plantarum LM14-1	9.42 ± 0.02	8.12 ± 0.15*	6.59 ± 0.11	4.22 ± 0.65*
L. plantarum LM14-2	9.44 ± 0.03	7.93 ± 0.08*	6.87 ± 0.11	5.08 ± 0.04*,**
L. plantarum LM15-1P	9.39 ± 0.05	7.89 ± 0.26*	6.53 ± 0.54	4.74 ± 0.23*
L. plantarum LM15-2	9.38 ± 0.06	7.83 ± 0.13*	5.98 ± 0.70	3.76 ±0.14*,**
L. pentosus PD3-1	9.40 ± 0.03	7.85 ± 0.33*	6.67 ± 0.19	5.03 ± 0.02*
En. lactis PD3-2	9.29 ± 0.03	6.01 ± 0.63*,**	5.31 ± 0.73	3.33 ± 0.54*,**
L. pentosus PD9-2	9.06 ± 0.06	7.76 ± 0.15*	5.91 ± 0.75	4.47 ± 0.31*
Lc. lactis subsp. *lactis* NH2-7C	8.56 ± 0.07	8.05 ± 0.14*	7.00 ± 0.15	4.76 ± 0.25*
L. rhamnosus GG	9.38 ± 0.04	7.78 ± 0.18*	6.68 ± 4.83	4.83 ± 0.13*

[a]Results indicate mean ± SD of three independent experiment.
*, showed the significant difference of the comparison between initial time and gastric/small intestinal-emptying time ($P \leq 0.05$). **, showed the significant difference of the comparison between selected strains and *L. rhamnosus* GG ($P \leq 0.05$).

Subsequently, all selected strains were transited and incubated in small intestinal condition at pH 7 for 5 h. The viability of all selected strains showed a reduction of the log from 1.35 to 3 compared with the log CFU in intestinal condition at 0 h. Notably, the excellent viability of *L. plantarum* LM14-2 was potentially higher than the probiotic *L. rhamnosus* GG in this study. Furthermore, the viability of *L. pentosus* PD3-1 was similar to the probiotic *L. rhamnosus* GG.

This finding revealed that *L. plantarum* LM14-2 and *L. pentosus* PD3-1could retain viability when exposed to the gastrointestinal condition. Furthermore, the attractive viability of strain LM14-2 was supported by the *in silico* analysis of strain LM14-2 that demonstrated the essential genes involved in the stress responses, such as acid and bile salt (Table 25). These genes are vital in maintaining pH between intra- and extra-bacterial cytoplasm. Consequently, they could be the potential probiotic candidate.

4.6 Adhesion assay

Adhesion ability is essential for probiotics to colonize and provide beneficial effects (i.e., cholesterol assimilation and immunomodulation activity) (Guan et al., 2020; Krausova et al., 2019). Caco-2 cell line *in vitro* models for probiotic adherence

studies have been widely used to screen putative probiotic cultures (Bernet et al., 1994; Chauviere et al., 1992; Elo et al., 1991; Tuomola et al., 1999; Wang et al., 2008). The adhesion ability of selected strains is shown in Figure 16. *L. plantarum* LM14-2 (82.46%) showed the greatest adherence rate, followed by *L. plantarum* LM6-2 (76.49%), Adherence ability to Caco-2 cells of *L. plantarum* LM14-2 was significantly higher than those of *L. pentosus* PD3-1 (72.54%), *L. plantarum* LM15-2 (69.01%), *L. plantarum* LM6-1 (66.96%), *Lc. lactis* subsp. *lactis* NH2-7C (63.03%), *L. plantarum* LM7-2-2B (62.09%), *L. pentosus* PD9-2 (58.63%), *L. plantarum* LM14-1 (50.14%), *L. plantarum* LM15-1P (49.19%), *L. plantarum* LM12-1 (44.92%), and *En. lactis* PD3-2 (31.16%).

Remarkably, *L. plantarum* LM14-2 showed the adhesion ability with no statistically significant difference compared to *L. rhamnosus* GG (81.71%). From the *in silico* analysis of strain LM14-2 (Table 25), the great adhesion ability was also supported by the presence of potential genes encoding responsible for adhesion adhesive ability. The adhesion ability of selected strains in this study is consistent with earlier studies (Duary et al., 2011; García-Cayuela et al., 2014; Thamacharoensuk et al., 2017).

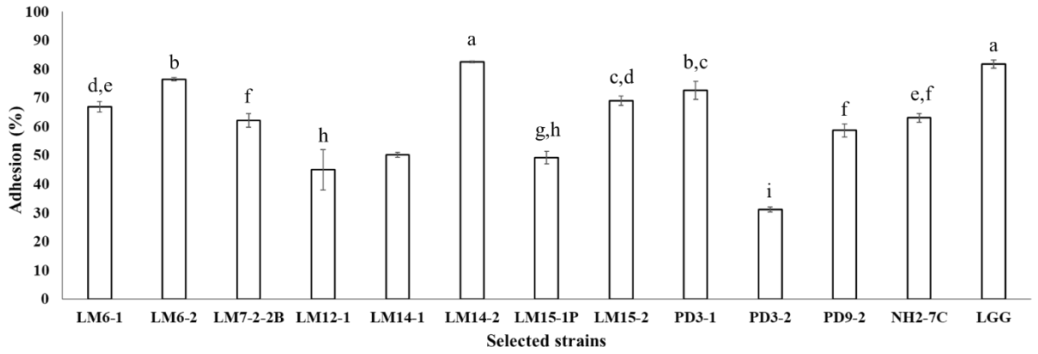


Figure 16 The adhesion ability of selected strains to Caco-2 cells

Data represent the mean ± SD. The different alphabets mean significant difference ($P \leq 0.05$)

Furthermore, this research could indicate that the adhesion ability of selected strains to Caco-2 cells was strain-specific and varied within the same species (Duary et al., 2011). The adhesion of selected strains varies among strains, depending on the

cell surface properties such as hydrophobicity and extracellular protein profiles. These selected strains exhibited excellent capability and might be potential candidate probiotics for further *in vivo* studies to assess their extra health-promoting benefits because they could colonize in the gut.

4.7 Immunomodulation effects

The immunomodulatory effects of the selected strains are shown in Table 13 and Figure 17. For IL-12 induction in simulated intestinal condition (Caco-2 and RAW264.7 cells), *En. lactis* PD3-2 had the greatest ability to induce IL-12 production (57.45 ± 7.22 ng/mL), and followed by *L. plantarum* LM12-1 (53.12 ± 6.43 ng/mL) and *L. plantarum* LM15-1P (51.78 ± 4.72 ng/mL). Conversely, *L. plantarum* LM6-1 and *L. pentosus* PD3-1 showed the lowest inducibility of IL-12 at 7.15 ± 1.22 and 7.72 ± 2.85 ng/mL, respectively. The IL-12 induction ability of LAB in this study is in accordance with other studies (Chen et al., 2013; Noriyuki Iwabuchi et al., 2012; Thamacharoensuk et al., 2017).

Table 13 Immunomodulatory effects of the selected strains

Species/ strain no.	IL-12 (ng/ml)	IFN-γ (ng/ml)	hBD2 (relative value)	NO (μM)
L. plantarum LM6-1	7.15 ± 1.22*	21.84 ± 6.64	2.26 ± 0.20*	17.89 ± 0.05**
L. plantarum LM6-2	20.62 ± 4.82	49.25 ± 18.21	1.91 ± 0.23*	13.52 ± 0.28**
L. plantarum LM7-2-2B	9.97 ± 3.92*	35.42 ± 11.44	0.98 ± 0.11	16.65 ± 0.08**
L. plantarum LM12-1	53.12 ± 6.43*	59.93 ± 16.02	1.67 ± 0.25	16.64 ± 0.05**
L. plantarum LM14-1	9.21 ± 3.15*	31.01 ± 8.57	1.50 ± 0.10*	17.76 ± 0.17**
L. plantarum LM14-2	35.49 ± 6.85	44.89 ± 14.61	2.15 ± 0.07*	19.98 ± 0.28**
L. plantarum LM15-1P	51.78 ± 4.72*	27.40 ± 4.63	1.58 ± 0.04*	15.75 ± 0.14**
L. plantarum LM15-2	24.77 ± 3.42	35.91 ± 8.79	1.61 ± 0.06*	16.03 ± 0.39**
L. pentosus PD3-1	7.72 ± 2.85*	32.91 ± 5.79*	2.06 ± 0.27*	18.07 ± 0.25**
En. lactis PD3-2	57.45 ± 7.22*	53.88 ± 13.80*	1.96 ± 0.10*	8.30 ± 0.09**
L. pentosus PD9-2	15.38 ± 4.93*	33.95 ± 7.93*	2.45 ± 0.25*	19.13 ± 0.20**
No stimulation	29.52 ± 5.87	43.23 ± 12.72	1.00 ± 0.00	Not detected
LPS (positive control)	Not determined			32.47 ± 0.14

Results are expressed as means ± SD.

* $P < 0.05$, compared to no stimulation within each column; ** $P < 0.05$, compared to LPS (positive control).

For IFN-γ induction in simulated intestinal condition (Caco-2 and RAW264.7 cells), *L. plantarum* LM12-1 had the highest ability to stimulate IFN-γ production (59.93 ± 16.02 ng/mL) and followed by *En. lactis* PD3-2 (53.88 ± 13.80 ng/mL),

while *L. plantarum* LM6-1 had the lowest stimulated ability (21.84 ± 6.64 ng/mL). The IFN-γ induction in this study is consistent with similar the previous studies (Ou et al., 2011; Yamane et al., 2018). Besides, the beneficial effect of IFN-γ and IL-12 are important cytokines for antitumor effect (Kato et al., 1999).

For hBD2 production in simulated intestinal condition (Caco-2 and RAW 264.7 cells), almost selected strains could enhance hBD2 production, but *L. plantarum* LM7-2-2B suppressed it. The result of hBD2 stimulation in this study is in accordance with the previous studies (Kobatake & Kabuki, 2019; Schlee et al., 2008; Wehkamp et al., 2004) Therefore, this study suggests that numerous non-pathogenic probiotic bacteria, including lactobacilli and others, stimulate innate immunity via defensin induction. Additionally, the stimulation of defensins by probiotics such as lactobacilli might be an attractive new therapeutic approach for enhancing innate defense systems (Schlee et al., 2008).

For nitric oxide (NO) production in RAW264.7 cells, NO is a multifunctional mediator that plays an important role in the inflammation. The physiologically NO production in phagocytic cells is advantageous to the host's defense against pathogens and tumor cells. According to the NO production results, all the selected strain could variably stimulate NO production at a wide range of rates. The maximum NO production was found in *L. plantarum* LM14-2 (19.98 ± 0.28 µM) followed by *L. pentosus* PD9-2 (19.13 ± 0.20 µM), *L. pentosus* PD3-1 (18.07 ± 0.25 µM), *L. plantarum* LM6-1 (17.89 ± 0.05 µM) and *Lb. plantarum* subsp. *plantarum* LM14-1 (17.76 ± 0.17 µM). The NO induced production of LAB in this study is consistent with previous studies (Kmonickova et al., 2012; Korhonen et al., 2001; Surayot et al., 2014; Tejada-Simon & Pestka, 1999).

For the *Lc. lactis* subsp. *lactis* NH2-7C

Heat-killed cells

Lc. lactis subsp. *lactis* NH2-7C reduced the TNF-α (2,777.31 ± 45.29 pg/ml) and slightly suppressed IL-6 (86.30 ± 4.86 pg/ml) production as compared to the level of TNF-α (3,345.51 ± 93.91 pg/ml) and IL-6 (90.65 ± 0.78 pg/ml) in cell (no stimulation) experiment. However, the combination of heat-killed NH2-7C cells and LPS synergistically induced the TNF-α (4773.97 ± 221.42 pg/ml) and IL-6 (488.59 ± 11.57 pg/ml) production as compared to the level of TNF-α (4,158.97 ± 204.11 pg/ml) and IL-6 (270.65 ± 29.17 pg/ml) in cell (stimulation) experiment.

From these findings, it could be concluded that the stimulation of LPS primarily influenced the raising of both pro-inflammatory cytokines. The immunomodulatory effects of strains NH2-7C are shown in Figure 17.

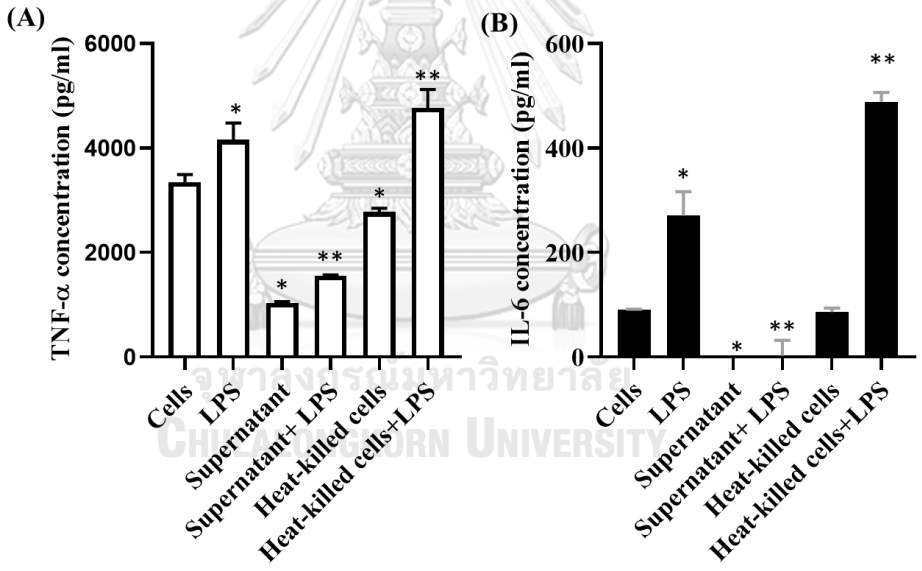


Figure 17 The immunomodulation effects of strain NH2-7C; The concentration of TNF-α (A) and IL-6 (B)

$^{*}P < 0.05$, compared to no stimulation within each column; $^{**}P < 0.05$, compared to LPS

Supernatant

The cell-free supernatant of strain NH2-7C showed remarkably anti-inflammatory activity by suppressing TNF-α and IL-6 production. The CFS suppressed the production of the TNF-α (1039.74 ± 14.22 pg/ml) and IL-6 (-46.52 ± 4.86 pg/ml) as compared to the level of TNF-α (3,345.51 ± 93.91 pg/ml) and IL-6 (90.65 ± 0.78 pg/ml) in the cells (no stimulation). In addition, the combination of cell-free supernatant and LPS showed the reduction of TNF-α (1556.03 ± 11.58 pg/ml) and IL-6 (2.50 ± 18.96 pg/ml) production as compared to the level of TNF-α (4,158.97 ± 204.11 pg/ml) and IL-6 (270.65 ± 29.17 pg/ml) in the cells (stimulation) experiment. These findings could be summarized as the cell-free supernatant of strain NH2-7C significantly lowered the production of both pro-inflammatory cytokines.

Consequently, these selected strains contained immunomodulatory effects (Kang, Kim, Park, et al., 2021; Kato et al., 1999). Remarkably, the non-viable cells of selected strains still contained immunomodulatory effects; hence, the benefits of dead/inactive cells of probiotics include a lower risk of probiotic sepsis and antibiotic resistance, as well as a longer shelf-life because there is no need to maintain the cold chain to preserve the viability of the probiotics (Shripada et al., 2020; Zendeboodi et al., 2020). Furthermore, this study revealed that bacterial strains, even though they belonged to the same species, could have various functional properties (Kang, Kim, Kim, et al., 2021). Some selected strains greatly stimulated the production of pro-inflammatory cytokines; consequently, these selected strains should be carefully monitored and applied appropriately. Cardiovascular diseases represent a complex group of clinical syndromes caused by various of interacting pathological factors. They include the most extensive disease population and rank first in all-cause mortality worldwide. Accumulating evidence demonstrates that cytokines play critical roles in the presence and development of cardiovascular diseases. Coronary heart disease (CHD), atherosclerotic heart disease (AHD), and congestive heart failure (CHF) are correlated to elevated levels of proinflammatory cytokines (i.e., IFN-γ, IL-6, and TNF-α). These cytokines play an important role in the developing of atherosclerotic plaque (Amin et al., 2020). In addition, IL-12 is a cytokine that regulates various of biological effects; it is closely related to the progression of various cardiovascular diseases, including atherosclerosis, hypertension, aortic

dissection, cardiac hypertrophy, myocardial infarction, and acute cardiac injury (Ye et al., 2020).

To our knowledge, this is the first *in vitro* investigation demonstrating selected strains isolated from Thai fermented foods used were more efficient in modulating the immune system.

4.8 The antimicrobial activity of selected strains

From all the selected strains, only *Lc. lactis* subsp. *lactis* NH2-7C showed antimicrobial activity using *L. sakei* JCM 1157^{T} (sensitive strain). Thus, this strain was selected to investigate antimicrobial compounds, such as growth and antimicrobial peptide production dynamics, partial purification, antimicrobial spectra, characterization, and bacteriocin synthesis cluster gene. In addition, some probiogenomic characteristics were analyzed.

The characteristics of *Lc. lactis* subsp. *lactis* NH2-7C

The strain NH2-7C was Gram-stain-positive, facultatively anaerobic, non-motile, cocci shape (Figure 18).

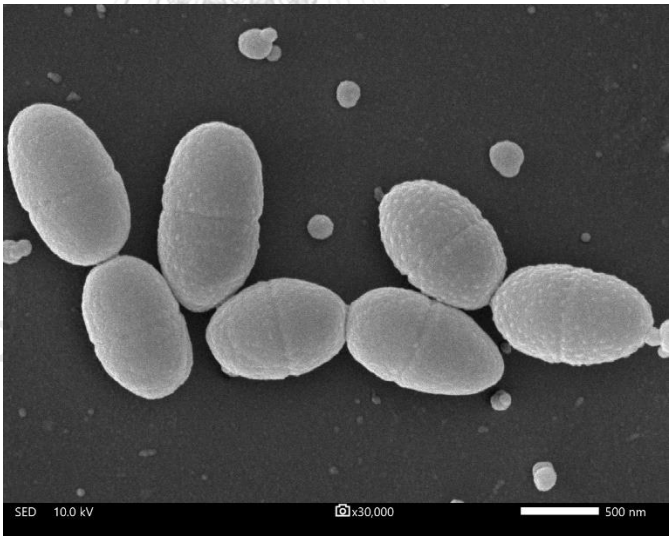


Figure 18 Scanning electron micrograph of strain NH2-7C grown on MRS agar at 30°C for 3 days

Growth occurred at 15–37 °C, pH 3.0-8.0 and in the presence of 1–8% (w/v) NaCl. It did not produce gas from glucose. NH2-7C produced L-lactic acid from glucose. Negative for catalase, and nitrate reduction; while positive for arginine

hydrolysis. The strain NH2-7C contained no *meso*-DAP in the cell wall. In API 50 CH is described in Table 14.

Based on full 16S rRNA gene sequence, the strain NH2-7C (1,551 bp) was closely related to *Lc. lactis* subsp. *lactis* JCM 5805^{T} with 99.80% similarity.

Table 14 The characteristics of strain NH2-7C

Characteristics of strain NH2-7C	
Cell shape	Cocci
Catalase	-
Gas from glucose	-
Growth in 6%	+
Growth in 8%	+
Growth at pH 3	+
pH 9	-
Growth at 15 Cº	+
45 Cº	-
Arginine hydrolysis	+
Nitrate reduction	-
Acid from:	
Glycerol (GLY)	-
Erythritol (ERY)	-
D-Arabinose (D-ARA)	-
L-Arabinose (L-ARA)	+
D-Ribose (RIB)	+
D-Xylose (DXYL)	+
L-Xylose (LXYL)	-
D-Xylose (ADO)	-
Methyl-beta-D-xylopyranoside (MDX)	-
D-Galactose (GAL)	+
D-Glucose (GLU)	+
D-Fructose (FRU)	+
D-Mannose (MNE)	+
L-Sorbose (SBE)	+
L-Rhamnose (RHA)	-
Dulcitol (DUL)	-
Inositol (INO)	-
D-Mannitol (MAN)	+
D-Sorbitol (SOR)	-
Methyl-alpha-D-mannopyranoside (MDM)	-
Methyl-alpha-D-glucopyranoside (MDG)	-
N-Acetylglucosamine (NAG)	+
Amygdalin (AMY)	+
Arbutin (ARB)	+
Esculin ferric citrate (ESC)	+

Characteristics of strain NH2-7C	
Salicin (SAL)	+
D-Cellobiose (CEL)	+
D-Maltose (MAL)	+
D-Lactose (bovine origin; LAC)	+
D-Melibiose (MEL)	-
D-Saccharose (sucrose; SAC)	+
D-Trehalose (TRE)	+
Inulin (INU)	-
D-Melezitose (MLZ)	-
D-Raffinose (RAF)	-
Amidon (starch; AMD)	W
Glycogen (GLYG)	-
Xylitol (XLT)	-
Gentiobiose (GEN)	W
D-Turanose (TUR)	-
D-Lyxose (LYX)	-
D-Tagatose (TAG)	-
D-Fucose (DFUC)	-
L-Fucose (LFUC)	-
D-Arabitol (DARL)	-
L-Arabitol (LARL)	-
Potassium gluconate (GNT)	W
Potassium 2-ketogluconate (2KG)	-
Potassium 5-ketogluconate (5KG)	-
Isomer of lactic acid	L
meso-DAP	-

+, positive reaction; w, weakly positive reaction; -, negative reaction

Whole-Genome sequence of *Lc. lactis* subsp. *lactis*

The genomic features of *Lc. lactis subsp. lactis* NH2-7C (accession no. JAIWQY000000000) were characterized using whole-genome sequencing and comprehensive bioinformatic analysis (Table 15), leading to a circular genome map construction (Figure 19). The draft genome sequence of strain NH2-7C was 2,576,236 bp, with a genomic DNA G + C content of 34.9%, N_{50} of 177,851, L_{50} of 5, and genome coverage of 178×. CheckM showed 98.98% genome completeness and 0.55% contamination. The Prokaryotic Genome Annotation Pipeline annotation identified 2,606 of total genes, of which 2,512 are protein-coding genes, 38 of total pseudo genes, 3 (2 (5s), 2 (16s), 2 (23S)) rRNAs, 46 tRNA genes, and 4 non-coding RNA (ncRNA) genes; while, the Rapid Annotation of microbial genomes using Subsystems Technology (RAST) identified 2,665 (No. of CDSs) and 49 RNA genes. Furthermore,

DDBJ Fast Annotation and Submission Tool (DFAST) did not detect CRISPRS in strain NH2-7C, and circular genomic and the subsystem were constructed and shown in Figure 19.

Table 15 Genomic features of *Lc. lactis* subsp. *lactis* NH2-7C

Attribute	**Strain NH2-7C**
Isolation source	Nham (a Thai traditional fermented pork)
Accession no.	JAIWQY000000000
Genome quality:	
Genome quality	Good
Coarse consistency	99.8
Fine consistency	99.1
Genome size (bp)	2,576,236
G+C content (%)	34.9%
Genome coverage	178x
N50	177,851
L50	5
No. of contigs	73
No. of subsystems	242
No. of coding sequences	2,665
No. of RNAs	49
No. of CRISPR	0

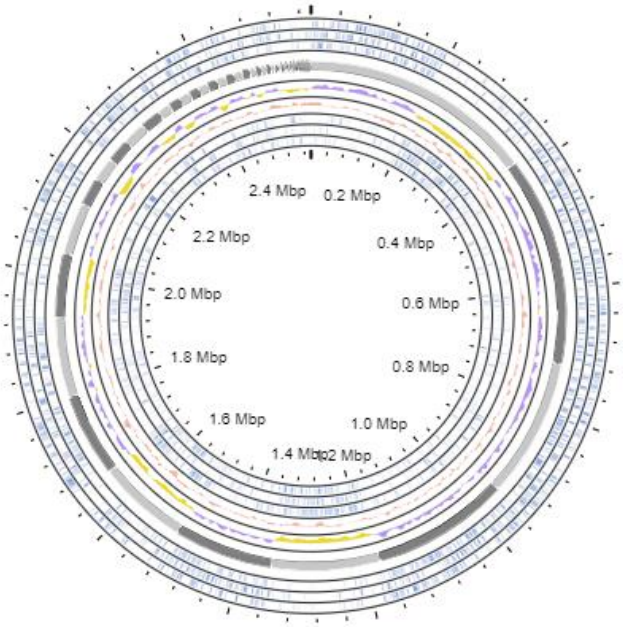


Figure 19 Circular genomic map of Lc. lactis subsp. lactis NH2-7C

The information is indicated as follows: open reading frames (ORFs) (blue), GC skew (+) (green), GC skew (-) (yellow), and GC content (pink)

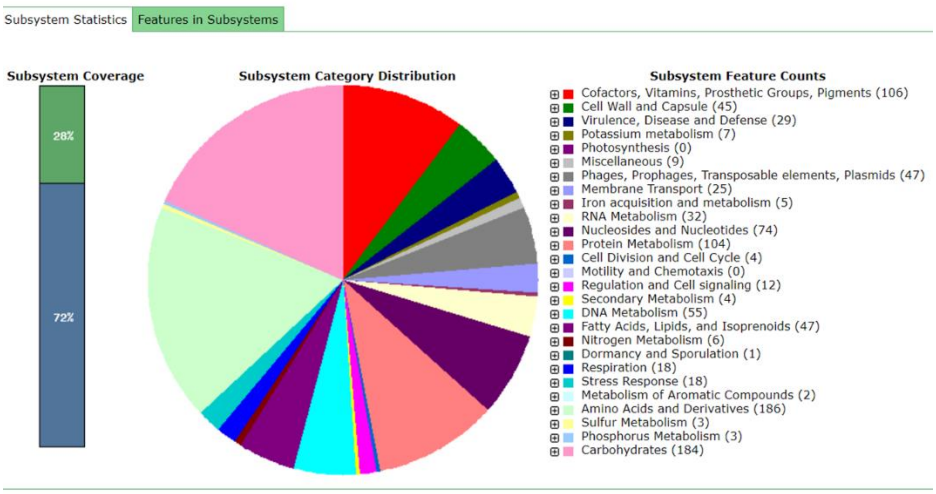


Figure 20 An overview of the subsystem categories assigned to the genome of *Lc. lactis* subsp. *lactis* NH2-7C. The genomic sequence of the strain NH2-7C was annotated using the Rapid Annotation System Technology (RAST) server. The pie chart demonstrates the count of each subsystem feature and the subsystem coverage

4.9 The confirmation proteinaceous characteristic of antimicrobial compound produced from *Lc. lactis* subsp. *lactis* NH2-7C

The CFS (pH 4.32) of strain NH2-7C showed an inhibitory effect on *L. sakei* JCM 1157^{T} (indicator strain). Thus, the CFS NH2-7C was selected and treated with several proteolytic enzymes such as trypsin, α-chymotrypsin, proteinase K, and pepsin. The effect of proteolytic enzymes on the antimicrobial activity of CFS is shown in Figure 21. The result showed that the antimicrobial activity of CFS NH2-7C was partially and mostly inactivated by proteolytic enzymes, indicating that it contained a proteinaceous structure as a bacteriocin (Alvarez-Cisneros et al., 2010), and this finding is in the accordance with the study of Lash et al. (2005).

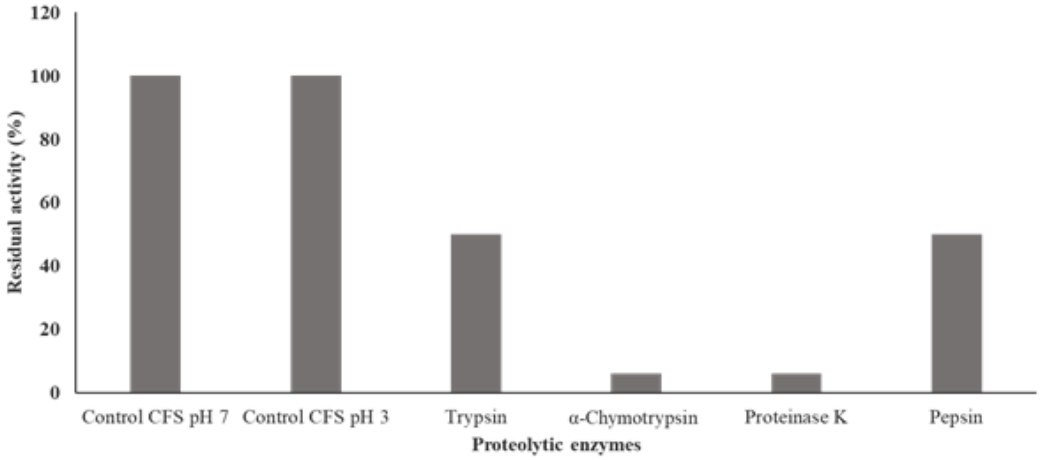


Figure 21 Effects of proteolytic enzymes on antimicrobial activity of CFS NH2-7C

4.10 Time course bacteriocin production of strain NH2-7C

In MRS broth at 30°C, the growth, bacteriocin production, and pH dynamics of *Lc. lactis* subsp. *lactis* NH2-7C were investigated (Figure 22). During growth, the NH2-7C strain initiated an exponential growth phase at 4 h, maintained a stationary phase from 8 to 20 h, and began the death phase 24 h later. Bacteriocin activity against the indicator strain (*L. sakei* JCM 1157^{T}) was evaluated. Therefore, bacteriocin was produced at 4 h during the initial exponential growth phase and reached its highest activity at 20 h during the late stationary phase with an activity of 51,200 AU/ml. The results indicated that the bacteriocin production was more evident and raised during the exponential growth phase, followed by a reduction during the end of the stationary phase (Gaspar et al., 2018). The reduction of antimicrobial activity found after a prolonged incubation period could be due to the degradation of

the bacteriocin by proteolytic enzymes, changes in the environmental conditions (Elayaraja et al., 2014; Huang et al., 2009), adsorption on producing strain (Xie et al., 2009) or bacteriocin aggregation (Collins et al., 1993). A reducing trend in the pH was observed while the growth proceeded and dropped to pH 4.32 from the initial pH 6.10 of the culture medium.

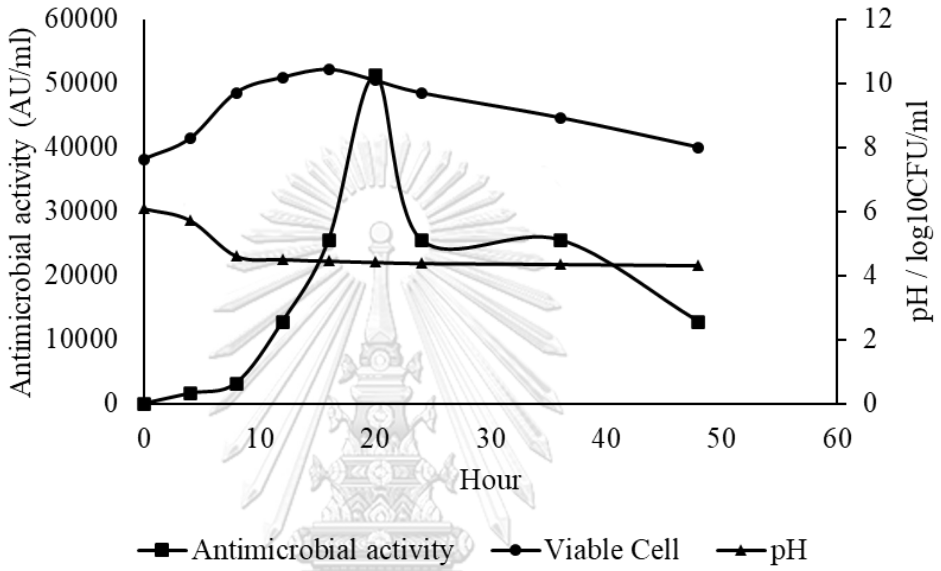


Figure 22 Time course bacteriocin production of strain NH2-7C

4.11 Partial purification of antimicrobial peptide

The antimicrobial peptide of NH2-7C was partially purified by AmberiteXAD-16 and Sp-sepharose. Since the maximum amount of bacteriocin was produced at 20 h, the culture supernatant was harvested at 20 h of incubation and then was used for bacteriocin purification. A two-step purification procedure was carried out, including hydrophobic interaction and cation-exchange chromatography. Fifty percent of the activity in the culture supernatant was recovered by hydrophobic interaction chromatography (Amberlite XAD-16). After being subjected to cation-exchange chromatography (SP-sepharose), the bacteriocin activity was recovered in a 0.25 M NaCl fraction. Finally, approximately 10% of the total activity of the culture supernatant was obtained after these purification steps. The total antimicrobial activity, yield and the purification fold of the bacteriocin, and the purification procedure are summarized in Table 16.

Table 16 Partial purification of bacteriocin produced by *Lc. lactis* subsp. *lactis* NH2-7C

Fraction	Volume (ml)	Activity (AU/ml)	Total activity (AU)	Yield (%)	Total protein (mg)	Specific activity (AU/mg)
Culture supernatant	1,000	51,200	5.12×10^{7}	100	1.51×10^{4}	3.4×10^{3}
Amberlite	500	51,200	2.56×10^{7}	50	2.67×10^{3}	9.6×10^{3}
Sp-sepharose	50	102,400	5.12×10^{6}	10	1.98×10^{2}	2.6×10^{4}

[a]Antimicrobial activity [in arbitary units (AU)] was assayed by spot-on-lawn method using *L. sakei* JCM 1157^{T}.
[b]The protein concentration (in mg/ml) was determined by the Lowry method.

4.12 Antimicrobial spectra

The antimicrobial spectra of bacteriocin NH2-7C are shown in Table 17. Among indicator strains used in this study, bacteria belonging to the LAB group were sensitive to bacteriocin NH2-7C. These findings is consistent with the fundamental characteristic of bacteriocin, which inhibits or generally kills closely related bacterial strains (Tiwari, 2022). Furthermore, bacteriocin NH2-7C demonstrated broad antimicrobial spectra against not only Gram-positive but also Gram-negative bacteria. Remarkably, several pathogens were inhibited including *L. innocua* ATCC 33090^{T}, *L. monocytogenes* ATCC 19115^{T}, *S. aureus* ATCC 6535^{T}, *St. mutans* DMST 18777^{T}, *St. suis* NaH, *Aeromonas hydrophila* B1 AhB1, *Helicobacter pylori* ATCC 43504^{T}, *H. pylori* 3875 (isolated from a patient suffering from gastritis), *H. pylori* BK 364 (isolated from a patient who has gastric cancer), *Vibrio harveyi* AQVH 01, *V. parahaemolyticus* AHPND 1691 (strain containing toxin plasmid), and *V. parahaemolyticus* AHPND 1681 (strain no containing toxin plasmid). However, *Candida albican* ATCC 10231^{T} and *C. albican* ATCC 90028^{T} were resistant to the inhibitory effects of bacteriocin NH2-7C. From the results, bacteriocin NH2-7C was effective against various clinical pathogens and the *Lc. lactis* subsp. *lactis* NH2-7C showed desirable characteristics. Furthermore, the anti-*H. pylori* activity of partially purified bacteriocin NH2-7C was supported by the study of Kim et al. (2003), which reported the nisin A contained anti-*H. pylori* activity. Consequently, it may have a potential probiotic application.

Table 17 Antimicrobial spectra of strain NH2-7C

Indicator strain	**Antimicrobial activity (AU/ml)**
Gram-positive bacteria:	
B. circulans JCM 2504^T	N.A.
B. subtilis JCM 1465^T	200
B. cereus ATCC 6633^T	100
En. faecalis JCM 5803^T	400
En. faecium JCM 5804^T	2,400
Lb. plantarum ATCC 14917^T	400
Lb. sakei JCM 1157^T	3,200
Lc. lactis ATCC 19435^T	400
Ln. mescenteroides JCM 6124^T	2,400
Li. inocua ATCC 33090^T	100
Li. monocytogenes ATCC 19115^T	200
M. luteus MIII	100
M. luteus NBRC 12708^T *(Kocuria rhizophila)*	150
P. dextrinicus JCM 5887^T	6,400
P. pentosaceus JCM 5885^T	400
S. aureus ATCC 23235^T	N.A.
Methilcillin-resistant *S. aureus* DMST 20635	N.A.
S. aureus ATCC 25923^T	N.A.
S. aureus ATCC 6538	200
S. aureus Cowan I	400
S. aureus DMST 6512^T	N.A.
Streptococcus agalactiae 1611	200
St. gordonii DMST 35778^T	N.A.
St. iniae SI 1810	N.A.
St. mutans DMST 18777^T	400
St. pyogenes DMST 17020^T	N.A.
St. suis NaH	400
St. suis P1/7	400

N.A. = no antimicrobial activity

Table 17 Antimicrobial spectra of strain NH2-7C

Indicator strain	Antimicrobial activity (AU/ml)
Gram-negative bacteria:	
A. hydrophila B1 AhB1	400
C. coli NCTC 11353^T	N.A.
E. coli ATCC 25922	N.A.
E. coli O157:H7	N.A.
E. coli F18	N.A.
E. coli ATCC 35401	N.A.
E. coli JCM 1093	N.A.
H. pylori ATCC 43504^T	6,400
H. pylori 3875	3,200
H. pylori BK 364	3,200
Ps. aeroginosa ATCC 27853^T	N.A.
*Sa. T*yphimurium ATCC 13311^T	N.A.
Vibrio algenolyticus Va	N.A.
V. harveyi AQVH 01	200
V. parahaemolyticus DMST 26792^T	N.A.
V. parahaemolyticus with AHPND toxin plasmid 1691	800
V. parahaemolyticus without AHPND toxin plasmid 1681	800
V. vulnificus 1809	N.A.
Yeast & Mold:	
Candida albicans ATCC 10231^T	N.A.
Candida albicans ATCC 90028^T	N.A.

N.A. = no antimicrobial activity

4.13 Characterization of bacteriocin NH2-7C

The effect of various enzymes, pH, temperature, and chemicals on antimicrobial activity

The antimicrobial activity of partially purified bacteriocin NH2-7C was affected by hydrolytic enzymes, chemicals, pH and temperature (Table 18). The partially purified bacteriocin NH2-7C was mostly inactivated by protease-K, α-chymotrypsin, lipase, amylase, trypsin, and partially inactivated by pepsin. This suggested the proteinaceous nature of the inhibitory compound (Gupta & Tiwari, 2015). The effects of pH and heat treatment on antimicrobial activity of partially purified bacteriocin NH2-7C was investigated. The partially purified bacteriocin

NH2-7C was stable under the temperature treatment at 100°C for 10, 20, 30 min; conversely, the activity decreased by 50% on applying sterilization temperature (121 °C for 15 min). Thus, the partially purified bacteriocin NH2-7C was found to be thermostable. For the effects of pH, the partially purified bacteriocin NH2-7C was found to be active over a wide pH range between 2 and 13. At the pH between 2 and 7 the activity was stable; while the activity decreases were obtained from pH 9 to 13. As a common feature of bacteriocins, partially purified bacteriocin NH2-7C was stable in the wide range of pH and temperature (Papagianni, 2003; Papagianni & Papamichael, 2011). However, partially purified bacteriocin NH2-7C seemed to be more stable in acidic condition. These finding are consistent with the previous studies of (Moreno et al., 2000; ŞAnlibaba et al., 2009). The inactivation of bacteriocin activity by proteases also provided safety since this bacteriocins could be degraded by enzymes in the human gastrointestinal tract, making them ineffective against beneficial microorganisms. For the effects of chemicals, the partially purified bacteriocin NH2-7C with organic solvents including ethanol, isopropanol, and acetonitrile did not cause any loss of antimicrobial activity. The antimicrobial activity of partially purified bacteriocin NH2-7C was not affected by 1% (v/v) Tween 20, Tween 80, Triton X-100 EDTA; while, the exposition of this compound to 1% urea and resulted in the reduction of the bacteriocin activity. The enhanced activity of partially purified bacteriocin NH2-7C with SDS was observed. Because SDS acts as a destabilizer that alters the permeability of the cell membrane of sensitive bacteria, it consequently increases the susceptibility to bacteriocin (Alakomi et al., 2000; Shafa & Salton, 1960). The sensitivity of partially purified bacteriocin NH2-7C to chemicals were similar to the other bacteriocins (Wang et al., 2018; Woraprayote et al., 2015). Nonetheless, it appears that the sensitivity to surfactants and urea is reliant on bacteriocin (Todorov et al., 2010; Woraprayote et al., 2015).

Table 18 Effects of enzymes, chemicals, pH and temperatures on antimicrobial of partially purified bacteriocin NH2-7C

Treatment	Relative activity (%) after treatment[a]
	Partially purified bacteriocin NH2-7C
Untreated	100.00
Enzymes:	
Trypsin	6.25
α-Chymotrypsin	3.13
Pepsin	50.00
Protease-K	1.56
Lipase	3.13
Amylase	3.13
Organic solvents:	
Ethanol	100.00
Isopropanol	100.00
Acetonitrile	100.00
Chemicals:	
Tween 20	100.00
Tween 80	100.00
SDS	200.00
EDTA	100.00
Triton-X-100	100.00
Urea	50.00
pH:	
2	100.00
3	100.00
5	100.00
7	100.00
9	12.50
11	6.25
13	1.56
Temperatures:	
100 °C, 10 min	100.00
100 °C, 20 min	100.00
100 °C, 30 min	100.00
121 °C, 15 min	50.00

[a]The activity of an untreated sample was defined as 100%

4.14 The bacteriocin synthesis cluster gene

Bacteriocin mining, through BAGEL v.4.0., identified bacteriocinogenic genetic cluster in the genome of strain NH2-7C and it is shown in Figure 23. The result showed the presence of a gene cluster encoding the production of nisin A (*nisABTCIPRKFEG*). This cluster includes 11 genes (*nisA*, organized into four

operons: *nisA* (nisin structural gene), *nisBTCIP* (nisin maturation, immunity, and transport), *nisRK* (nisin regulation), and *nisFEG* (nisin immunity). The nisin A cluster genes are shown in Figure 23. Of these genes, the *nisA* gene encodes nisin A precursor peptide composing of 57-amino acid residues, containing 23-amino acid residues, N-terminal leader peptide that is associated with directing the alteration and targeting process of nisin precursor *nisB* and *nisC* encode membrane-associated proteins involved in the intracellular post-translational modification reaction. *nisT* encodes a putative transporter protein of the ABC translocator family involved in the translocation of the fully modified nisin precursor across the cytoplasmic membrane. *nisP* encodes a subtilisin-like protease involved in extracellular proteolytic activation. Two systems involved in immunity to nisin of the producing cell are derived from *nisI* and *nisFEG*. *nisI* encodes a lipoprotein involved in the self-protection of the producing bacterium against nisin, and *nisFEG* encodes a putative ABC exporter involved in nisin extrusion. *nisR* and *nisK* encode a response regulator and a sensor kinase of the histidine protein kinase family, respectively, that belong to a class of two-component regulatory systems (Cheigh & Pyun, 2005). Based on the *in silico* analysis, it could be possibly concluded that the antimicrobial peptide of strain NH2-7C was identified as nisin A with a Bit-score of 115.16 and 100% identity. The presence of nisin cluster genes in *Lactococcus* sp. is consistent with the previous studies (Nguyen & Kim, 2018; Zhao et al., 2015).

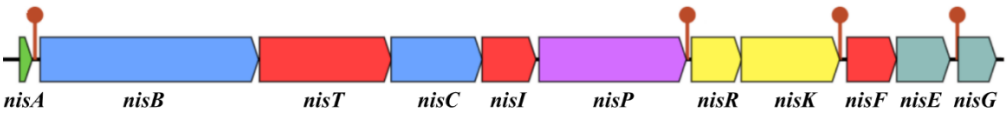


Figure 23 The gene cluster encoding the production of nisin A in strain NH2-7C using the online BAGEL v.4.0. web-based tool (http://bagel4.molgenrug.nl/index.php, accessed on 18 June 2022). Terminators are shown as maroon line circle ends

4.15 Probiotic genetic markers of *Lc. lactis* subsp. *lactis* NH2-7C

The essential characteristics of microorganisms that meet the criteria as probiotic strains are their survival mechanisms and adaption ability in harsh environments. (Nguyen & Kim, 2018). Probiotics contain genes that promote tolerance to undesirable conditions (Nguyen & Kim, 2018). The genomic data of *Lc. lactis* subsp. lactis NH2-7C discovered genes coding for stress response, adhesion, metabolic rearrangement, lactate synthesis and transcriptional regulators, all of which contribute to survival in acidic pH and prevention of pathogen colonization in a gut environment (Table 19). The presence of adhesins in the probiotic cell wall is essential to the ability to adhere to the gut (Monteagudo-Mera et al., 2019). The adhesive genes were detected. Muñoz-Provencio et al. (2012) proposed that sortase-dependent surface proteins have a role in mucosal adhesion processes and in certain aspects of intestinal homeostatic maintenance. Sortase class A (srtA) is involved for the LPXTG proteins covalently to the cell wall. Some LPXTG proteins, especially those with mucus-binding domains, contribute to adherence to host surfaces (Marraffini et al., 2006). Mucus-binding proteins contribute to the adherence of bacteria to the intestinal mucosa (Hymes et al., 2016; Lehri et al., 2015). A total of 31 genes responsible for acid and bile salt stress were identified (Table 19). The F1F0-ATPase is encoded by the atp operon, which comprises the following genes: *atpC, atpD, atpG, atpH, atpF, atpB, atpE,* and *atpA* (Ventura et al., 2004). The atp genes are vital for the survival or tolerance of acidic environments. The "atp" operon is primarily related to the pumping of protons, consequently contributing to maintaining neutral pH (Duary et al., 2010). S-Ribosylhomocysteinase (*luxS*) play an important role in the autoinducer-2 synthesis. It has been observed that the Autoinducer-2 promotes stress resistance (Liu et al., 2018). The *luxS* gene is also associated to the capacity to adhere to intestinal epidermal cells (Jia et al., 2018). Additionally, moonlighting protein genes, or multifunctional protein genes, such as elongation factor Tu and chaperonin GroEL, have been associated with adhesion to epithelial cells and immunomodulation (Abriouel et al., 2017). Furthermore, probiotics perform an essential function in the host gut by synthesizing micronutrients and factors such as amino acids, fatty acids, oligosaccharides, vitamins, and enzymes. Overall, the draft genome information of *Lc. lactis* subsp. *lactis* NH2-7C will help us

to comprehend the molecular basis of for its probiotic effects properly. They may assist its future applications.

Table 19 The potential genes associated to various probiotic characteristics from *Lc. lactis* subsp. *lactis* NH2-7C genome

Putative function	Genes	Gene product
Modulation of immune system / Acid stress	*clpB*	Potential immunogenic proteins
	lspA	Lipoprotein signal peptidase
	tuf	Elongation factor Tu
Nutritional synthesis and several essential processes	*ccpA*	Catabolite control protein A
Cholesterol-lowering effect / Bile resistance	*bsh*	Choloylglycine hydrolase
Adhesion or interaction with the host	*ylcC*	Class A sortase
	dltD	D-alanyl-lipoteichoic acid biosynthesis protein DltD
	dltA	D-alanylation of LTA
	lspA	Lipoprotein signal peptidase
	tuf	Elongation factor Tu
	mtsA	Manganese ABC transporter substrate-binding protein
	eno2	Enolase 2
	gapB	Type I glyceraldehyde-3-phosphate dehydrogenase
	groS	Co-chaperonin GroES
	groL	Chaperonin GroEL
	pgi	Glucose-6-isomerase
Acid stress	*atpC*	ATP synthase subunit epsilon
	atpD	ATP synthase subunit beta
	atpA	ATP synthase subunit alpha
	atpG	ATP synthase subunit gamma
	atpH	ATP synthase subunit delta
	atpF	ATP synthase subunit B
	atpB	ATP synthase subunit A
	atpE	ATP synthase subunit C
	recA	Protein RecA (recombinase A)
	relA	GTP pyrophosphokinase
	groS	Co-chaperonin GroES
	groL	Chaperonin GroEL
	aspS	Aspartate-tRNA ligase
Acid stress/Bile resistance	*dnaK*	Chaperone protein DnaK
	dnaJ	Chaperone protein DnaJ
	glmU	Bifunctional UDP-N acetylglucosamine diphosphorylase/glucosamine phosphate
	luxS	S-ribosylhomocysteine lyase
	gadB	Glutamate decarboxylase; GABA transporter
Bile resistance	*nagB*	Glucosamine-6-phosphate

Putative function	Genes	Gene product
		deaminase
	pyrG	CTP synthase
	argS	Arginine-tRNA ligase
	rpsC	30S ribosomal protein S3
	rpsE	30S ribosomal protein S5
	rplD	50S ribosomal protein L4
	rplE	50S ribosomal protein L5
	rplF	50S ribosomal protein L6
DNA and protein protection and repair	*msrB*	Peptide methionine sulfoxide reductase MsrB
Fatty acid synthesis	*fabD*	Malonyl CoA-acyl carrier protein transacylase
	fabF	3-oxoacyl-[acyl-carrier-protein] synthase II
	fabI	Enoyl-[acyl-carrier-protein] reductase [NADH]
	accC	acetyl-CoA carboxylase biotin carboxylase subunit
Transcriptional regulator	*ctsR*	Transcriptional regulator CtsR
	hrcA	Heat-inducible transcriptional repressor HrcA
Matabolic rearrangement	*aldB*	Alpha-acetolactate decarboxylase

4.16 Safety assessment of strain NH2-7C

For strain proposed for probiotic application, genomic sequences are necessary for comprehensive safety assessments (Wassenaar et al., 2015). In addition to evaluating the genome for probiotic ability, strains should also be analyzed for genes associated with virulence, pathogenicity, and toxicity (Wassenaar et al., 2015). These genomic findings demonstrate the probiotic potential of strain NH2-7C. The PathogenFinder result (Table 20) demonstrated that *Lc. lactis* subsp. *lactis* NH2-7C was predicted as non-human pathogen. From the genome annotation, genes related to virulence factors were detected. Two genes are associated with defense, such as exopolysaccharides biosynthesis protein (*tagL_1* and *tagL_2*) and capsular polysaccharide biosynthesis protein. Exopolysaccharides are involved in cell adhesion on abiotic and biotic surfaces (Caro-Astorga et al., 2020). It assists bacteria withstand osmotic, desiccation, and oxidative stress environments (Y. Liu et al., 2017). Polysaccharides contribute to identifying isolate-specific characteristics vital for probiotic function, including stress resistance, adhesion, and the host's defense system (Lebeer et al., 2009). Capsular polysaccharides have been observed in the colonization of the gastrointestinal tract, and they regulate the immune system (Porter et al., 2017). In addition, the hemolysin III gene (*hlyIII*) was found in the genome of

the strain NH2-7C. Remarkably, the gene was also observed in various commercial probiotics, including an accepted Generally Recognized as Safe (GRAS) probiotic strain *L. plantarum* 299V, and a widely used commercial probiotic in China, *L. rhamnosus* GG. Hemolysis test using sheep-blood agar demonstrated a non-hemolysis. Generally, the hemolysin III gene is prevalent in *Lactobacillus* spp., and the strains containing the gene have been proven safe and are commercially available worldwide. The bacterium containing this gene should not pose a safety risk, provided that no other pathogenesis genes are observable in the genome. Nevertheless, the absence of other pathogenesis mechanisms, these virulence genes could be deemed as advantageous to the bacterium because they enhance bacterial endurance, may be beneficial in conditions where viable bacteria are required (e.g., starters and probiotics), and are also observed in the genomes of several commensal bacteria (Ho Sui et al., 2009). The primary issue concerning AMR genes in beneficial non-pathogenic bacteria is their transfer possibility to other pathogens, which may cause serious problems, lowering the efficiency of antibiotic treatment. To determine this risk, this study focused on two kinds of mobile elements: plasmids and bacteriophages because they are the most plausible vehicles for inter-cellular genetic exchange by transformation/conjugation and transduction, respectively. The strain NH2-7C contained one plasmid, indicating that NH2-7C is capable of self-transmission through conjugative transfer. Plasmid-associated antibiotic resistance is not very common among LAB, but it does occur, and safety implications should be taken into consideration. Checking the ability of a proposed probiotic strain to act as a donor for conjugative antibiotic resistance genes may be a sensible precaution in some instances (Salminen et al., 1998). For the presence of bacteriophage, the PHASTER tool (Arndt et al., 2016; Zhou et al., 2011) identified nine prophage regions (6 incomplete and 3 questionable) and the none of the ARGs (antibiotic resistance genes) were located in the prophage regions. The presence of phage regions may contribute to the acquirement of antibiotic resistance, the ability to survive in a new environment, the improvement of adhesion ability, or even to turning the bacteria pathogenic (Casjens, 2003). However, the prophage regions in the NH2-7C genome were six incomplete (10 – 60 of score) and questionable (75 – 80 of score). In addition, *Lc. lactis* subsp. *lactis* NH2-7C contained tetracycline-resistance gene (*tet*(S)). The presence of

tetracycline-resistance characteristic was usually observed in several probiotic *Lactococcus* strains and other probiotics in previous studies (Ma et al., 2021; Ramalho et al., 2019). In this study, the presence of *tetS* gene is consistent with the *in vitro* antibiotic resistance to tetracycline (30 µg). Thus, these issues could be unconcerned (Oliveira et al., 2017). In conclusion, the analyses of the safety, survival and probiotic aspects of *Lc. lactis* subsp. *lactis* NH2-7C emphasized the potential use of this strain as a target for the future development of LAB probiotics.

Table 20 Pathogenicity prediction, prophage detection and antibiotic resistance genes (ARGs) analysis from PathogenFinder of CGE and PHASTER (Default program settings applied)

Attribute/Strain	***Lc. lactis* subsp. lactis NH2-7C**	***L. plantarum* 299v**	***L. rhamnosus* GG**
Probability of being a human pathogen	0.209	0.185	0.198
Input proteome coverage (%)	1.74	0.48	40.5
Matched pathogenic families	0	0	0
Matched not pathogenic families	44	15	1147
Conclusion	Non-human pathogen	Non-human pathogen	Non-human pathogen
No. of plasmid	1 (rep32, 99.7% identity)	2 (rep28, 98.17% identity; rep38, 99.0% identity)	0
No. of phage	9	4	5
Antibiotic resistance genes (ARGs)			
CARD:			
- No. of perfect hits	0	0	0
- No. of strict hits	1 (*tet*(s))	0	0
- No. of loose hits	155	194	207
ResFinder	*tet*(S)	No resistance	No resistance

4.17 The candidate probiotic strain LM14-2

Based on the presence of BSH activity and the great cholesterol assimilation, the strain LM14-2 was also selected for the probiogenomic analysis.

(I) The characteristics and phylogenomic analysis of *L. plantarum* LM14-2

The strain LM14-2 was Gram-stain-positive, facultatively anaerobic, non-motile, straight rods. Growth occurred at 15–45 °C, pH 3.0-8.0 and in the presence of 1–8% (w/v) NaCl. It did not produce gas from glucose. LM14-2 produced DL-lactic acid from glucose. Catalase are negative. Arginine hydrolysis and nitrate reduction

are negative. The strain LM14-2 contained *meso*-DAP in the cell wall. In API 50 CH is described in Table 21.

Table 21 The characteristics of strain LM14-2

Characteristics of strain LM14-2	
Cell shape	Rod
Catalase	-
Gas from glucose	-
Growth in 6%	+
Growth in 8%	+
Growth at pH 3	+
pH 9	-
Growth at 15 Cº	+
45 Cº	+
Arginine hydrolysis	-
Nitrate reduction	-
Acid from:	
Glycerol (GLY)	W
Erythritol (ERY)	W
D-Arabinose (D-ARA)	W
L-Arabinose (L-ARA)	+
D-Ribose (RIB)	+
D-Xylose (DXYL)	W
L-Xylose (LXYL)	W
D-Xylose (ADO)	W
Methyl-beta-D-xylopyranoside (MDX)	W
D-Galactose (GAL)	+
D-Glucose (GLU)	+
D-Fructose (FRU)	+
D-Mannose (MNE)	+
L-Sorbose (SBE)	W
L-Rhamnose (RHA)	+
Dulcitol (DUL)	W
Inositol (INO)	W
D-Mannitol (MAN)	+
D-Sorbitol (SOR)	+
Methyl-alpha-D-mannopyranoside (MDM)	-
Methyl-alpha-D-glucopyranoside (MDG)	-
N-acetylglucosamine (NAG)	+
Amygdalin (AMY)	+
Arbutin (ARB)	+
Esculin ferric citrate (ESC)	+
Salicin (SAL)	+
D-Cellobiose (CEL)	+
D-Maltose (MAL)	+

Characteristics of strain LM14-2	
D-Lactose (bovine origin; LAC)	+
D-Melibiose (MEL)	+
D-Saccharose (sucrose; SAC)	+
D-Trehalose (TRE)	+
Inulin (INU)	-
D-Melezitose (MLZ)	+
D-Raffinose (RAF)	+
Amidon (starch; AMD)	-
Glycogen (GLYG)	-
Xylitol (XLT)	+
Gentiobiose (GEN)	W
D-Turanose (TUR)	-
D-Lyxose (LYX)	-
D-Tagatose (TAG)	-
D-Fucose (DFUC)	-
L-Fucose (LFUC)	-
D-Arabitol (DARL)	-
L-Arabitol (LARL)	-
Potassium gluconate (GNT)	+
Potassium 2-ketogluconate (2KG)	-
Potassium 5-ketogluconate (5KG)	-
Isomer of lactic acid	DL
meso-DAP	+

+, positive reaction; w, weakly positive reaction; -, negative reaction

Based on full 16S rRNA gene sequence, the strain LM14-2 (1,567 bp) was closely related to *L. plantarum* ATCC 14917^T, *L. argentoratensis* DSM 16365^T, *L. pentosus* DSM 20314^T, and *L. paraplantarum* DSM 10667^T with 100%, 100%, 99.93% and 99.73% similarity, respectively. The study found that analysis based only on the 16 S rRNA gene could not be used to accurately identify bacterial species in this group, especially *L. plantarum* and *L. argentoratensis*.

A phylogenomic tree analysis (Figure 24), LM14-2 was formed the same cluster within several *L. plantarum* strains (Meier-Kolthoff & Göker, 2019). Strain LM14-2 was closely related to *L. plantarum* DSM 20174^T or ATCC 14917^T with a digital DNA–DNA hybridization (dDDH) value of 93.7%, which was the highest dDDH value observed with closely related species (Table 22). The dDDH value was greater than the recommended cut-off points of 70% for species delineation (Auch et al., 2010). The values of ANIb, ANIm between strain LM14-2 and *L. plantarum* DSM

20174^{T} or ATCC 14917^{T} were 98.2 % and 99.27 %, respectively. The mentioned ANI values were above the species boundary value (ANI > 95–96%) (Lee et al., 2016). For these reasons, the results of characteristics and whole genome analysis supported strain LM14-2 as representing a member of *Lactiplantibacillus plantarum*.

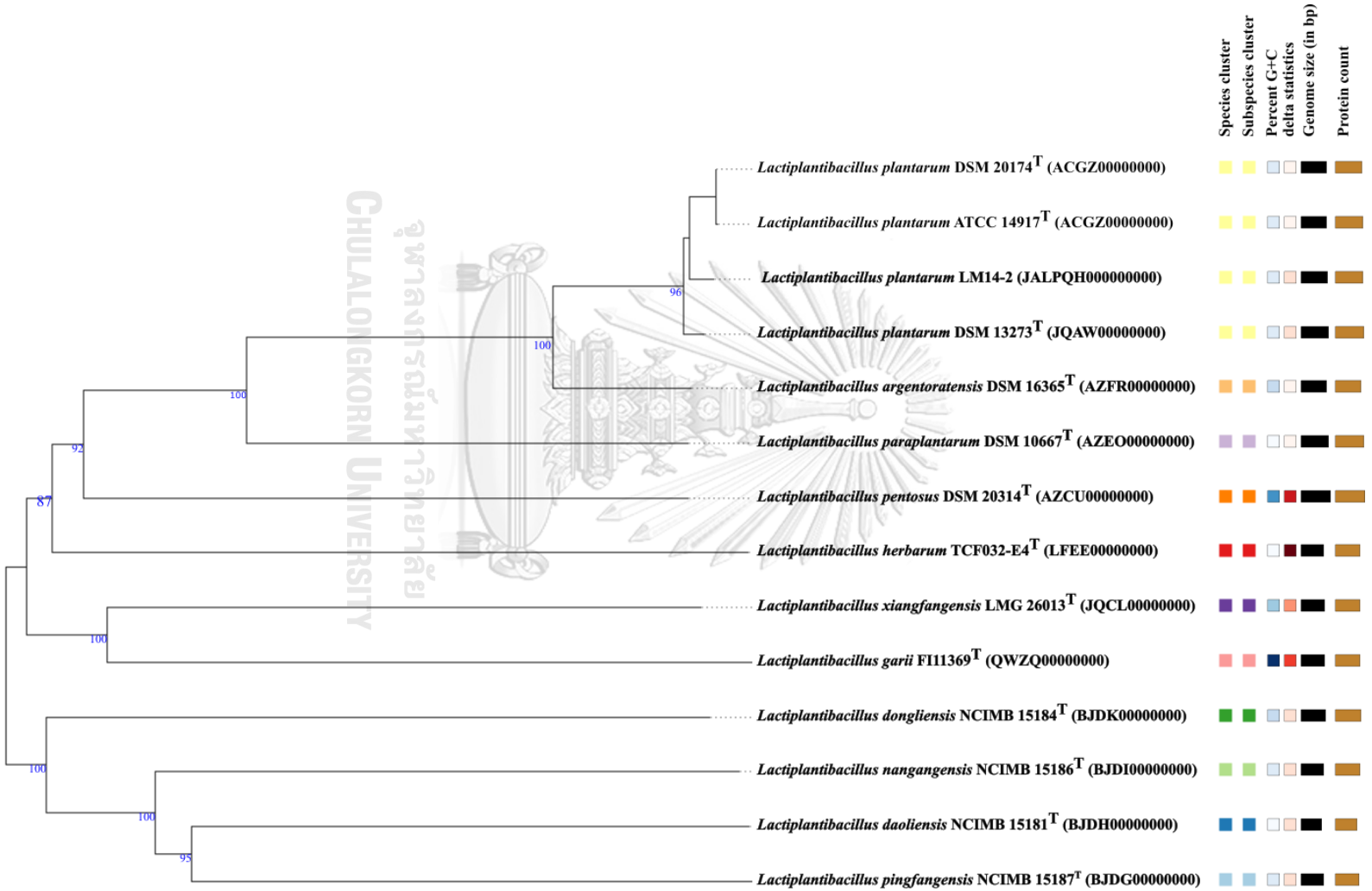

Species cluster
Subspecies cluster
Percent G+C delta statistics
Genome size (in bp)
Protein count
Lactiplantibacillus plantarum DSM 20174T (ACGZ00000000)
Lactiplantibacillus plantarum ATCC 14917T (ACGZ00000000)
Lactiplantibacillus plantarum LM14-2 (JALPQH000000000)
Lactiplantibacillus plantarum DSM 13273T (JQAW00000000)
Lactiplantibacillus argentoratensis DSM 16365T (AZFR00000000)
Lactiplantibacillus paraplantarum DSM 10667T (AZEO00000000)
Lactiplantibacillus pentosus DSM 20314T (AZCU00000000)
Lactiplantibacillus herbarum TCF032-E4T (LFEE00000000)
Lactiplantibacillus xiangfangensis LMG 26013T (JQCL00000000)
Lactiplantibacillus garii FI11369T (QWZQ00000000)
Lactiplantibacillus dongliensis NCIMB 15184T (BJDK00000000)
Lactiplantibacillus nangangensis NCIMB 15186T (BJDI00000000)
Lactiplantibacillus daoliensis NCIMB 15181T (BJDH00000000)
Lactiplantibacillus pingfangensis NCIMB 15187T (BJDG00000000)
96
100
100
92
87
100
100
100
95

Figure 24 Phylogenomic tree based on whole genome sequence data result of strain LM14-2 and closely related type strains reconstructed on the Type (Strain) Genome Server (TYGS)

Table 22 ANIb and ANIm (%) and the digital DNA-DNA hybridization (dDDH) values between the draft genomes of the strain LM14-2; *L. plantarum* DSM 20174^{T} (=ATCC 14917^{T}); *L. plantarum* DSM 13273^{T}; *L. argentorensis* DSM 16365^{T} and *L. paraplantarum* DSM 10667^{T}

Genomic data: 1, *L. plantarum* LM14-2 (JALPQH000000000); 2, *L. plantarum* DSM 20174^{T} (=ATCC 14917^{T}) (ACGZ00000000); 3, *L. plantarum* DSM 13273^{T} (JQAW00000000); 4, *L. argentoraensis* DSM 16365^{T} (AZFR00000000); and 5, *L. paraplantarum* DSM 10667^{T} (AZEO00000000)

Query genome	Reference genome	ANIb	ANIm	% dDDH (Formular 2*)	Model C.I. (%)	Distance	Prob. DDH >= 70%	G+C difference
1	2	98.92	99.27	93.7	91.9-95.1	0.0081	96.88	0.22
1	3	98.77	99.16	92.3	90.3-94.0	0.0096	96.52	0.02
1	4	94.78	95.66	62.6	59.7-65.4	0.0472	60.6	0.75
1	5	85.31	88.33	31.3	28.9-33.8	0.1354	0.18	0.57

*Recommended formula (identities/HSP length), which is liberated of genome length and is thus prosperous against the use of draft genome.

(II) Whole-Genome sequence of *L. plantarum* LM14-2

The genomic features of *L. plantarum* LM14-2 (accession no. JALPQH000000000) and other probiotics (*L. plantarum* 299V, and *Lacticaseibacillus rhamnosus* GG (ATCC 53103)) were characterized using whole-genome sequencing and comprehensive bioinformatic analysis (Table 23), leading to a circular genome map construction (Figure 25).

Table 23 Genomic features of *Lactiplantibacillus plantarum* LM14-2, *L. plantarum* 299V, and *Lacticaseibacillus rhamnosus* GG (ATCC 53103)

Attribute	LM14-2	299V	GG (ATCC 53103)
Source	Thai fermented mussel (*Hoi-dong*)	Healthy human intestinal mucosa	Fecal samples of a healthy human
Accession no.	JALPQH000000000	LEAV00000000	FM179322
Genome size (bp)	3,311,812	3,302,055	3,010,111
Plasmids	0	2 (rep28, 98.17% identity; rep38, 99.0% identity)	0
Genome qualities:			
- Genome quality	Good	Good	Poor
- Completeness (%)	99.35	99.35	99.49
- Coarse consistency	97.9	98	99.4
- Fine consistency	96.1	96.2	98.3
G+C content (%)	44.3	44.4	46.7
Genome coverage	250x	48x	No data
N50	331,723	173,004	-
L50	4	8	1
No. of contig	40	67	1
No. of subsystem	230	232	231
No. of coding sequences	3,254	3,264	3,009
No. of RNA	67	60	72
No. of CRISPRS	0	0	1

The draft genome sequence of strain LM14-2 was 3,311,812 bp, with a genomic DNA G + C content of 44.3%, N_{50} of 331,723, L_{50} of 4, and genome coverage of 250×. CheckM showed 99.35% genome completeness and 2.35% contamination. The Prokaryotic Genome Annotation Pipeline annotation identified 3,115 predicted genes, of which 2,999 are protein-coding genes, 41 pseudo genes, 76 RNA genes, 70 tRNA genes, and 4 non-coding RNA (ncRNA) genes; while, the Rapid Annotation of microbial genomes using Subsystems Technology (RAST) identified 3,254 (No. of CDSs) and 67 RNA genes. Furthermore, DDBJ Fast Annotation and Submission Tool (DFAST) did not detect CRISPRS in strain LM14-2

and circular genomic and subsystem were constructed and shown in Figure 25 and Figure 26.

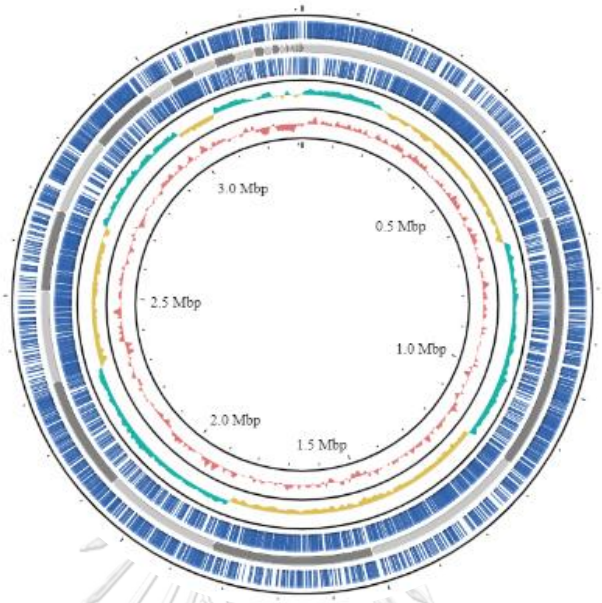


Figure 25 Circular genomic map of *L. plantarum* LM14-2

The information is indicated as follows: open reading frames (ORFs) (blue), GC skew (+) (green), GC skew (-) (yellow) and GC content (pink)

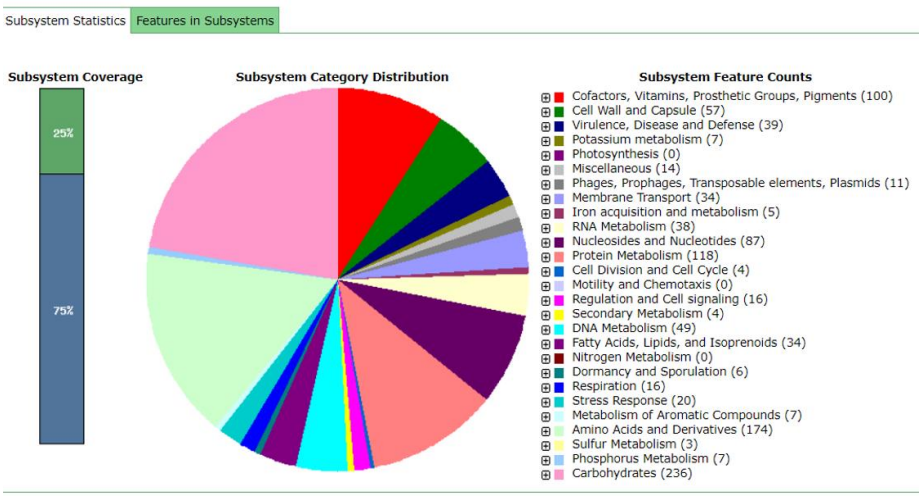


Figure 26 An overview of the subsystem categories assigned to the genome of *L. plantarum* LM14-2. The genomic sequence of the strain LM14-2 was annotated using the Rapid Annotation System Technology (RAST) server. The pie chart demonstrates the count of each subsystem feature and the subsystem coverage

4.18 Safety assessment

For strain proposed for probiotic application, genomic sequences are necessary for comprehensive safety assessments (Wassenaar et al., 2015). In addition to evaluating the genome for probiotic ability, strains should also be analyzed for genes associated with virulence, pathogenicity, and toxicity (Wassenaar et al., 2015). These genomic findings demonstrate the probiotic potential of strain LM14-2. The PathogenFinder result (Table 24) demonstrated that *Lb. plantarum* LM14-2 was predicted as non-human pathogen. From the genome annotation, genes related to virulence factors were detected. Two genes are associated with stress defense, such as exopolysaccharides biosynthesis protein (*cps2B* and *cps4B*) and capsular polysaccharide biosynthesis protein (*cpsE*). Exopolysaccharides are involved in cell adhesion on abiotic and biotic surfaces (Caro-Astorga et al., 2020). It assists bacteria withstand osmotic, desiccation, and oxidative stress environments (Y. Liu et al., 2017). Polysaccharides contribute to identifying isolate-specific characteristics vital for probiotic function, including stress resistance, adhesion, and the host's defense system (Lebeer et al., 2009). Capsular polysaccharides have been observed in the colonization of the gastrointestinal tract, and they regulate the immune system (Porter et al., 2017). In addition, the hemolysin III gene (*hlyIII*) was found in the genome of the strain LM14-2. Remarkably, the gene was also observed in various commercial probiotics, including an accepted Generally Recognized as Safe (GRAS) probiotic strain *L. plantarum* 299V, a widely used commercial probiotic in China, *L. rhamnosus* GG, (marketed and researched probiotic isolates) (Stage et al., 2020), and numerous other *Lactobacillus* strains in the GenBank. Hemolysis test using sheep-blood agar demonstrated a hazy zone of hemolysis around the growth of bacteria, similar to the zone surrounding the probiotic *L. rhamnosus* GG, indicating that the two strains had comparable hemolysis activity. This finding is consistent with previous research (Chokesajjawatee et al., 2020). Generally, the hemolysin III gene is prevalent in *Lactobacillus* spp., and the strains containing the gene have been proven safe and are commercially available worldwide. The bacterium containing this gene should not pose a safety risk, provided that no other pathogenesis genes are observable in the genome. Nevertheless, the absence of other pathogenesis mechanisms, these virulence genes could be deemed as advantageous to the bacterium because they enhance

bacterial endurance, may be beneficial in conditions where viable bacteria are required (e.g., starters and probiotics), and are also observed in the genomes of several commensal bacteria (Ho Sui et al., 2009). The primary issue concerning AMR genes in beneficial non-pathogenic bacteria is their transfer possibility to other pathogens, which may cause serious problems, lowering the efficiency of antibiotic treatment. To determine this risk, this study focused on two kinds of mobile elements: plasmids and bacteriophages because they are the most plausible vehicles for inter-cellular genetic exchange by transformation/conjugation and transduction, respectively. The strain LM14-2 did not contain any plasmid; consequently, no *oriT* was found in any of the plasmid, indicating that LM14-2 is incapable of self-transmission through conjugative transfer. For the presence of bacteriophage, the PHASTER tool (Arndt et al., 2016; Zhou et al., 2011) identified three prophage regions and none of the ARGs (antibiotic resistance genes) were located in the prophage regions. As a result of these findings, it was established that the absence of AGR genes in LM14-2 does not present a risk of transfer to other bacteria; therefore, the strain does not provide a safety issue for the functional and transferrable ARGs characteristic.

Table 24 Pathogenicity prediction, prophage detection and antibiotic resistance genes (ARGs) analysis from PathogenFinder of CGE and PHASTER (Default program settings applied)

Attribute/Strain	***L. plantarum* LM14-2**	***L. plantarum* 299v**	***L. rhamnosus* GG**
Probability of being a human pathogen	0.187	0.185	0.198
Input proteome coverage (%)	0.49	0.48	40.5
Matched pathogenic families	0	0	0
Matched not pathogenic families	15	15	1147
Conclusion	Non-human pathogen	Non-human pathogen	Non-human pathogen
No. of phage	3	4	5
Antibiotic resistance genes (ARGs)			
CARD:			
- No. of perfect hits	0	0	0
- No. of strict hits	0	0	0
- No. of loose hits	192	194	207
ResFinder	No resistance	No resistance	No resistance

4.19 Identification of genes associated stress response, microbe-host interactions and bacteriocin biosynthesis

The vital characteristics of microorganisms that meet the criteria as probiotic strain are their survival mechanisms and ability to adapt and survive in harsh environments. (Nguyen & Kim, 2018). Probiotics contain genes that promote tolerance to undesirable conditions (Nguyen & Kim, 2018). The genomic data of *L. plantarum* LM14-2 discovered genes coding for stress response, adhesion, metabolic rearrangement, lactate synthesis and transcriptional regulators, all of which contribute to survival in acidic pH and prevention of pathogen colonization in a gut environment (Table 25). The presence of adhesins in the probiotic cell wall is essential for adherent ability to the gut (Monteagudo-Mera et al., 2019). The adhesive genes were detected. Muñoz-Provencio et al. (2012) proposed that sortase-dependent surface proteins have a role in mucosal adhesion processes and in certain aspects of intestinal homeostatic maintenance. Sortase class A (*srtA*) is involved for the LPXTG proteins covalently to the cell wall. Some LPXTG proteins, especially those with mucus-binding domains, contribute to adherence to host surfaces (Marraffini et al., 2006). Mucus-binding proteins contribute to the adherence of probiotic to the intestinal mucosa (Hymes et al., 2016; Lehri et al., 2015) and *L. plantarum* LM14-2 harbored gene coding for mucus-binding protein *mub*. A total of 47 genes responsible for acid and bile salt stress were identified (Table 25). The F_1F_0-ATPase is encoded by the atp operon, which comprises the following genes: *atpC, atpD, atpG, atpH, atpF, atpB, atpE, and atpA* (Ventura et al., 2004). The atp genes are vital for the survival or tolerance of acidic environments. The "atp" operon is primarily related to the pumping of protons, consequently contributing to maintaining neutral pH (Duary et al., 2010). S-Ribosylhomocysteinase (*luxS*) play an important role in the Autoinducer-2 synthesis. It has been observed that the Autoinducer-2 promotes stress resistance (Liu et al., 2018). In addition, the *luxS* gene is also associated to the capacity to adhere to intestinal epidermal cells (Jia et al., 2018). Additionally, moonlighting protein genes, or multifunctional protein genes, such as elongation factor Tu and chaperonin GroEL, have been associated with adhesion to epithelial cells and immunomodulation (Abriouel et al., 2017). The draft genome information of *L. plantarum* LM14-2 will help us to comprehend the molecular basis of for its probiotic effects properly.

Table 25 Potential genes associated to various probiotic characteristics from *L. plantarum* LM14-2 genome

Putative function	Genes	Gene product
Modulation of immune system / Acid stress	*clpB*	Potential immunogenic proteins
	lspA	Lipoprotein signal peptidase
	tuf	Elongation factor Tu
Nutritional synthesis and several essential processes	*ccpA*	Catabolite control protein A
Cholesterol-lowering effect / Bile resistance	*bsh*	Choloylglycine hydrolase
Adhesion or interaction with the host	*srtA*	Class A sortase
	dltD	D-alanyl-lipoteichoic acid biosynthesis protein DltD
	dltA	D-alanylation of LTA
	mub	Mucus-binding protein
	glnH1	Glutamine ABC transporter substrate-binding protein
	lspA	Lipoprotein signal peptidase
	tuf	Elongation factor Tu
	mtsA	Manganese ABC transporter substrate-binding protein
	eno2	Enolase 2
	gapB	Type I glyceraldehyde-3-phosphate dehydrogenase
	groS	Co-chaperonin GroES
	groL	Chaperonin GroEL
	glnA	Glutamine synthase
	pgi	Glucose-6-isomerase
Acid stress	*atpC*	ATP synthase subunit epsilon
	atpD	ATP synthase subunit beta
	atpA	ATP synthase subunit alpha
	atpG	ATP synthase subunit gamma
	atpH	ATP synthase subunit delta
	atpF	ATP synthase subunit B
	atpB	ATP synthase subunit A
	atpE	ATP synthase subunit C
	recA	Protein RecA (recombinase A)
	relA	GTP pyrophosphokinase
	groS	Co-chaperonin GroES
	groL	Chaperonin GroEL
	aspS	Aspartate-tRNA ligase
Acid stress/Bile resistance	*gpmA1*	2,3-bisphosphoglycerate dependent phosphoglycerate mutase 1
	gpmA2	2,3-bisphosphoglycerate-dependent phosphoglycerate mutase 2
	dnaK	Chaperone protein DnaK
	dnaJ	Chaperone protein DnaJ
	glmU	Bifunctional UDP-N acetylglucosamine diphosphorylase/glucosamine phosphate
	luxS	S-ribosylhomocysteine lyase
	gadB	Glutamate decarboxylase; GABA

Putative function	Genes	Gene product
		transporter
Bile resistance	*nagB*	Glucosamine-6-phosphate deaminase
	pyrG	CTP synthase
	argS	Arginine-tRNA ligase
	rpsC	30S ribosomal protein S3
	rpsE	30S ribosomal protein S5
	rplD	50S ribosomal protein L4
	rplE	50S ribosomal protein L5
	rplF	50S ribosomal protein L6
DNA and protein protection and repair	*dps1*	DNA starvation/stationary phase protection protein
	dps2	DNA starvation/stationary phase protection protein
	msrB	Peptide methionine sulfoxide reductase MsrB
Fatty acid synthesis	*fabD*	Malonyl CoA-acyl carrier protein transacylase
	fabH1	3-oxoacyl-[acyl-carrier-protein] synthase III protein 1
	fabH2	3-oxoacyl-[acyl-carrier-protein] synthase III protein 2
	fabF	3-oxoacyl-[acyl-carrier-protein] synthase II
	fabI	Enoyl-[acyl-carrier-protein] reductase [NADH]
	accC1	acetyl-CoA carboxylase biotin carboxylase subunit
	accC2	acetyl-CoA carboxylase biotin carboxylase subunit
Lactate synthesis	*mdh*	Malate dehydrogenase
Transcriptional regulator	*ctsR*	Transcriptional regulator CtsR
	hrcA	Heat-inducible transcriptional repressor HrcA
Matabolic rearrangement	*aldB*	Alpha-acetolactate decarboxylase

Furthermore, genomic analysis of several *L. plantarum* strains have recently discovered the genetic loci responsible for bacteriocin production (Todorov, 2009). This study discovered that strain LM14-2 contained six genes that are crucial for the production of the bacteriocin: plantaricin NC8-α, F, E, J, NC8-β and, K with 98.60, 107.07, 112.46, 112.46, 112.85, 114.01 bit-score, respectively (Figure 27). However, *L. plantarum* LM14-2 lacked several genes, including *plnA, plnB, plnC, plnL, plnN, plnO, plnQ, plnR, plnT, plnU, plnV, plnW,* and *plnX* gene, which are also essential for bacteriocin production (Diep et al., 2009). Thus, it could be summarized that this strain could not produce antimicrobial compound. These findings demonstrated that LM14-2 contains probiotic properties. Genomic analysis of several *L. plantarum*

strains have recently discovered the genetic loci responsible for bacteriocin production (Todorov, 2009).

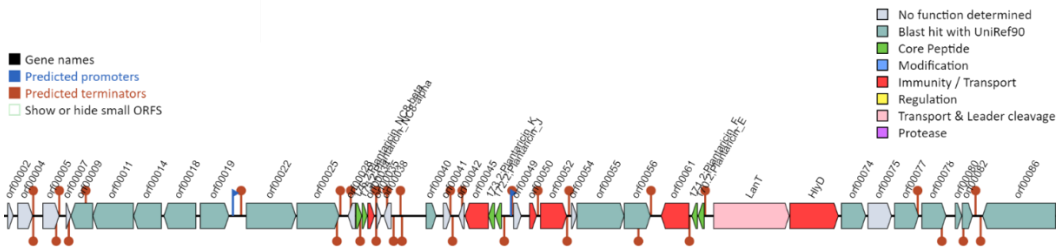


Figure 27 Genetic map of the bacteriocin synthetic genes of *L. plantarum* LM14-2. using the online BAGEL v.4.0. web-based tool (http://bagel4.molgenrug.nl/index.php, accessed on 18 June 2022). Terminators are shown as maroon line circle ends

4.20 Carbohydrate-active enzyme analysis

The presence of genes involved in carbohydrate metabolism in *L.* plantarum LM14-2 and *Lc. lactis* subsp. *lactis* NH2-7C is essential for its potential adaptability to the environment of the gut environment and its interaction with the human host, hence enhancing its survival, competitiveness, and longevity.

For strain LM14-2, the genome of strain LM14-2 comprised 101 carbohydrate-active enzyme genes, including 39 glycosyltransferase (GT) genes, 51 glycoside hydrolase (GH) genes, five carbohydrates esterase (CE) genes, and three carbohydrate-binding molecules (CBMs), and three auxiliary activities (AA).

For strain NH2-7C, the genome of strain NH2-7C comprised 85 carbohydrate-active enzyme genes, including 28 glycosyltransferase (GT) genes, 44 glycoside hydrolase (GH) genes, six carbohydrates esterase (CE) genes, and six carbohydrate-binding molecules (CBMs), and one auxiliary activity (AA).

Consequently, LM14-2 and NH2-7C could use several mono- and polysaccharides as energy sources and produce complex compounds. The results may support the nomadic nature of the strain, which is generally characteristic of *L. plantarum* strains (Martino et al., 2016). The study of the GH enzyme families in strain LM14-2 and NH2-7C showed the presence of GH13 and GH32, which have been described as key oligosaccharide-degrading enzymes. Oligosaccharides are

source of prebiotics, which are related to human gut health (Pokusaeva et al., 2011; Tarrah et al., 2020). Furthermore, GH families play essential roles in carbohydrate hydrolysis and their action as retaining enzymes involved in synthesizing oligosaccharides that may be preferentially utilized as prebiotics by *L. plantarum* LM14-2, *Lc. lactis* subsp. *lactis* NH2-7C, and other gastrointestinal probiotic bacteria (Abriouel et al., 2017). In addition, glycosyltransferases catalyze the transfer of sugars from activated donor molecules to specified acceptors, which is necessary for constructing surface structures recognized by host immune systems (Chung et al., 2018). Thus, the *L. plantarum* LM14-2 and *Lc. lactis* subsp. *lactis* NH2-7C containing the numerous GT genes could be a potential probiotic, especially for immunomodulation and pathogen prevention.

CHAPTER V
CONCLUSION

Ninety strains of lactic acid bacteria (LAB) isolated from fermented foods and bark of *Tamarindus indica* and identified using the phenotypic characteristics and 16S rRNA gene sequence analysis. For the fermented mussel (*Hoi-dong*) samples, they were identified as *Companilactobacillus formosensis* (10 strains), *Lentilactobacillus buchneri* (8 strains), *Lactiplantibacillus plantarum* subsp. *plantarum* (17 strains), *Lacticaseibacillus rhamnosus* (1 strain), *Pediococcus pentosaceus* (5 strains) and *Pediococcus acidilactici* (1 strain), *Enterococcus thailandicus* (2 strains), *Enterococcu hirae* (1 strain), *Enterococcu durans* (1 strain), *Lactococcus lactis* subsp. *lactis* (1 strain), *Lactococcus lactis* subsp. *hordinae* (1 strain), and *Leuconostoc lactis* (1 strain). For the fermented fish (*Pla-paeng-daeng*) samples, they were identified as *Lactiplantibacillus pentosus* (6 strains), *Lactiplantibacillus argentoratensis* (1 strain), *Limosilactobacillus fermentum* (2 strains), *Companilactobacillus pabuli* (4 strains), *Companilactobacillus farciminis* (5 strains), *Companilactobacillus futsaii* (6 strains), and *Enterococcus lactis* (1 strain). For the fermented pork (*Nham*) samples, they were identified as *Lactiplantibacillus pentosus* (4 strains), *Lactiplantibacillus argentoratensis* (2 strains), *Lacticaseibacillus paracasei* subsp. *tolerans* (1 strain), *Pediococcus pentosaceus* (7 strains), *Lactococcus lactis* subsp. *lactis* (1 strain).

Furthermore, strain BCM23-1^T was isolated from bark of *Tamarindus indica* collected from Chiang Mai province. This strain produced D-lactic acid from glucose. It grew at 20-45 °C (optimum, 30 °C), pH 3.5-9 (optimum, pH 7.0) and in the presence of 1-4 % (w/v) NaCl. The cell-wall peptidoglycan contained *meso*-diaminopimelic acid (A1γ). The major isoprenoid quinone was menaquinone 7 (MK-7). Polar lipids analysis revealed the presence of diphosphatidylglycerol, phosphatidylglycerol, an unidentified aminophospholipid, an unidentified phospholipid and an unidentified lipid. The predominant cellular fatty acids were anteiso-$C_{17:0}$, anteiso-$C_{15:0}$, and iso-$C_{16:0}$ when cultivated on GYP agar plates. The 16S rRNA gene sequence similarity between strain BCM23-1^T and *Terrilactibacillus laevilacticus* NK26-11^T was 98.3 %. The draft genome of BCM23-1^T was 3.24 Mb in

size and contained 3088 coding sequences with an *in silico* DNA G+C content of 37.1 mol%. The values of ANIb, ANIm and digital DNA-DNA hybridization between strain BCM23-1^T and *T. laevilacticus* NK26-11^T were 89.9, 90.8 and 40.4 %, respectively. The results of phenotypic and chemotaxonomic, 16S rRNA gene sequence similarity, and whole genome analyses support strain BCM23-1^T as representing a novel species of *Terrilactibacillus* for which the name *Terrilactibacillus tamarindi* sp. nov. is proposed. The type strain is BCM23-1^T (=LMG 31662^T=JCM 33748^T=TISTR 2841^T).

Only 12 strains, eight strains of *L. plantarum* (LM6-1, LM6-2, LM7-2-2B, LM12-1, LM14-1, LM14-2, LM15-1P, and LM15-2), two strains of *L. pentosus* (PD3-1 and PD9-2), and one strain of *Lc. lactis* subsp. *lactis* NH2-7C demonstrated bile salt hydrolase activity by forming opaque white colonies. In addition, one strain of *En. lactis* (PD3-2) demonstrated bile salt hydrolase activity by forming halos around colonies. The selected strains exhibited that the percentage of cholesterol assimilation varied from 27.40 % to 86.07 %. *L. plantarum* LM14-2 (86.07 ± 5.03%) showed the highest cholesterol assimilation rate, while *L. pentosus* PD9-2 (27.40 ± 2.00%).

Twelve selected strains were determined the viability during gastrointestinal transit. The reduction of viable cells was observed through the incubation period in simulated gastric condition. The survival of selected strains was decreased by about 0.5-3.2 log cycle after incubation in simulated gastric condition at pH 3.0 for 3 h. In simulated gastric condition, the viability of *Lc. lactis* subsp. *lactis* NH2-7C was slightly decreased from 8.56 ± 0.07 to 8.05 ± 0.14 (log_{10}CFU/ml). Subsequently, all selected strains were transited and incubated in small intestinal condition at pH 7 for 5 h. The viability of all selected strains showed a reduction of the log from 1.35 to 3 compared with the log CFU in intestinal condition at 0 h. Notably, *L. plantarum* LM14-2 and *L. pentosus* PD3-1 showed excellent viability. This finding revealed that *L. plantarum* LM14-2 and *L. pentosus* PD3-1 could retain viability when exposed to the gastrointestinal condition. Furthermore, all selected strains showed varied adhesion ability to Caco-2 cells from 82.46 to 31.16%

The heat-killed cells of *L. plantarum* (LM6-1, LM6-2, LM7-2-2B, LM12-1, LM14-1, LM14-2, LM15-1P, LM15-2), *L. pentosus* (PD3-1 and PD9-2), and *En.*

lactis (PD3-2) still contained the variably immunomodulation effects on the level of IL-12, IFN-γ, hbD2, and NO. *En. lactis* PD3-2 potentially induced the production of IL-12 at 57.45 ± 7.22 ng/ml, while *L. plantarum* LM6-1 decreased the production of IL-12 at 7.15 ± 1.22 ng/ml. For IFN-γ induction, *L. plantarum* LM12-1 had the highest ability to stimulate IFN-γ production at 59.93 ± 16.02 ng/ml. Conversely, *L. plantarum* LM6-1 had the lowest stimulated ability at 21.84 ± 6.64 ng/ml. For hBD2 production, almost selected strains could enhance hBD2 production, but *L. plantarum* LM7-2-2B suppressed it. For nitric oxide (NO) production, the maximum NO production was found in *L. plantarum* LM14-2 at 19.98 ± 0.28 µM; however, *En. lactis* PD3-2 showed the minimum NO production at 8.30 ± 0.09 µM. Furthermore, the heat-killed cells and cell-free supernatant of *Lc. lactis* subsp. *lactis* NH2-7C showed immunomodulatory effects. The combination of heat-killed cells of NH2-7C and LPS synergistically induced the TNF-α (4773.97 ± 221.42 pg/ml) and IL-6 (488.59 ± 11.57 pg/ml) production. On the contrary, the cell-free supernatant of strain NH2-7C showed remarkably anti-inflammatory activity by suppressing TNF-α and IL-6 production.

Only *Lc. lactis* subsp. *lactis* NH2-7C showed antimicrobial activity. The cell-free supernatant of strain NH2-7C was pH 4.32 and sensitive to protease enzymes. The result revealed that the antimicrobial compounds in the CFS contained proteinaceous structure as a bacteriocin. The bacteriocin showed the highest antimicrobial activity at 20 h with an activity of 51,200 AU/ml with *L. sake*i JCM 1157^{T} (sensitive indicator). The antimicrobial peptide of NH2-7C was partially purified by AmberiteXAD-16 and Sp-sepharose. Fifty percent of the activity in the culture supernatant was recovered by hydrophobic interaction chromatography (Amberlite XAD-16). After being subjected to cation-exchange chromatography (SP-sepharose), the bacteriocin activity was recovered in a 0.25 M NaCl fraction. Finally, approximately 10% of the total activity of the culture supernatant was obtained after these purification steps. The antimicrobial spectra of bacteriocin NH2-7C were shown the inhibition activity with closely related bacterial strains and almost Gram-positive bacteria. Some Gram-negative pathogens, such as *A. hydrophila*, *H. pylori*, *V. harveyi*, and *V. parahaemolyticus*, were inhibited. However, *Candida albicans* strains could not be inhibited. The characteristic of partially purified bacteriocin NH2-7C

was stable under the temperature treatment at 100°C for 10, 20, and 30 min; conversely, the activity decreased by 50% on applying sterilization temperature (121 °C for 15 min). For the effects of pH, the partially purified bacteriocin NH2-7C was active over a wide pH range between 2 and 13. The activity was stable at the pH between 2 and 7, while a decrease in activity was obtained from pH 9 to 13. However, partially purified bacteriocin NH2-7C seemed more stable in acidic conditions. For the effects of chemicals, the bacteriocin NH2-7C with organic solvents, including ethanol, isopropanol, and acetonitrile, did not cause any loss of antimicrobial activity. The antimicrobial activity of bacteriocin NH2-7C was not affected by 1% (v/v) Tween 20, Tween 80, Triton X-100 EDTA; while the exposition of this compound to 1% urea resulted in the reduction of the bacteriocin activity. The enhanced activity of bacteriocin NH2-7C with SDS was observed. The bacteriocin of strain NH2-7C was identified as nisin A based on the genomic analysis.

The probiogenomic characteristics of *L. plantarum* LM14-2 and *Lc. lactis* subsp. *lactis* NH2-7C showed that these strains were considered safe, and they did not contain virulence elements. In addition, they composed of various genes that play roles in acid and bile salt tolerance, adhesion, and other positive benefits. Therefore, the combination of *in vitro* and *in silico* analysis suggested that *L. plantarum* LM14-2 and *Lc. lactis* subsp. *lactis* NH2-7C is considered a potential probiotic because it exhibits health-promoting effects and probiotic characteristics.

The results from this study provided the distribution of LAB in Thai fermented foods and the bark of *Tamarindus indica*, which are attractive isolation sources of probiotic lactic acid bacteria. According to the results of screening cholesterol-lowering effects, and probiotic properties. Also, the probiogenomic data of strain NH2-7C and LM14-2 supported the desirable features. Thus, these strains can be considered excellent candidates for use as probiotics. However, further investigations in an *in vivo* model are required.

APPENDIX A
Culture media

All media were suspended with distilled water and sterilized by autoclaving at 121 ºC for 15 min. For determination of acid production from carbon sources, the media were sterilized at 110 ºC for 10 min.

1. MRS agar

Proteose peptone No.3	10.0	g
Beef Extract	10.0	g
Yeast Extract	5.0	g
Dextrose	20.0	g
Polysorbate 80	1.0	g
Ammonium Citrate	2.0	g
Sodium Acetate	5.0	g
Magnesium Sulfate	0.1	g
Manganese Sulfate	0.05	g
Dipotassium Phosphate	2.0	g
Agar	15.0	g
Distilled water	1.0	L

For MRS broth, prepared with the same ingredients without the agar.

2. Columbia blood agar base supplemented with 5% sheep blood

Pancreatic Digest of Casein	10.0 g
Proteose Peptone No. 3	5.0 g
Yeast Extract	5.0 g
Beef Heart, Infusion from 500 g.	3.0 g
Corn starch	1.0 g
Sodium Chloride	5.0 g
Agar	15.0 g
Distilled water	1.0 L

After autoclaving, placed the media in water bath with temperature of 55 ºC, then added with 50 ml of sheep blood and gentle mixed before pouring to the plates.

3. Brain heart infusion agar

Calf Brains, Infusion from 200 g	7.7 g
Beef Heart, Infusion from 250 g	9.8 g
Proteose Peptone	10.0 g
Dextrose	2.0 g
Sodium Chloride	5.0 g
Disodium phosphate	2.5 g
Agar	15.0 g
Distilled water	1.0 L

For brain heart infusion soft agar, prepared with the same ingredients but reduced agar from 15.0 g to 7 g per 1 L of distilled water. After cooling BHI soft agar to 55 ºC, added horse serum at 5% final concentration before use

4. Salt solution

$MgSO_4.7H_2O$	400	mg
$MnSO_4.5H_2O$	20	mg
$FeSO_4.7H_2O$ 2	0	mg
NaCl	20	mg
Distilled water	10	ml

6. L (+)-Arginine agar medium

Peptone	1.0	g
K_2HPO_4	0.3	g
NaCl	5.0	g
L-(+) Arginine hydrochloride	10	g
Phenol red	0.01	g
Agar	3.0	g
Distilled water	1.0	L
Adjust to pH 6.8		

7. Nitrate broth

Yeast extract	5.0	g
Peptone	10.0	g
KNO_3	1.0	g
NaCl	10	g
Distilled water	1.0	L
Adjust to pH 6.8		

APPENDIX B
Reagents and buffers

1. 0.85% NaCl

NaCl	0.85	g
Distilled water	100	ml

2. 1X Phosphate buffer saline (PBS buffer)

NaCl	8	g
KCl	0.2	g
Na_2HPO_4	1.44	g
KH_2PO_4	0.24	g
Distilled water	1	L
pH 7.4		

3. Nitrate reduction test reagents

Sulphanilic acid solution

Sulphanilic acid	0.8	g
5N Acetic acid	100	ml

N,*N*-dimethyl-1-naphthylamine solution

N,*N*-dimethyl-1-naphthylamine	0.5	g
5N Acetic acid	100	ml

4. Mixed indicator

Bromthymol blue	0.2	g
Neutral red	0.1	g
Ethanol	300	ml

5. PCR reaction mixture

	Stock	1 Volume (100 µl)
Forward Primer : 20F	10 pmol/µl	4
Reward Primer : 1530R	10 pmol/µl	4
10 x *Taq* buffer (NH_4SO_4 –$MgCl_2$)	10 x	10
dNTP	2.0 mM	2
$MgCl_2$	25 mM	8
Taq DNA polymerase	5 Unit/µl	0.5
Milli-Q water	-	66.5
Template	Undilute	5

6. 1X Tris-acetate (TAE) buffer

50X Tris-acetate (TAE) buffer	20	ml
Distilled water	980	ml

7. Ethidium bromide solution (10 mg/ml)

Ethidium bromide	1	g
Distilled water	100	ml

8. 0.8% Agarose gel

Agarose	0.8	g
Distilled water	100	ml

Melt the mixture with the microwave.

APPENDIX C
Reagents and buffers for partial purification

1. 20% Ethanol

Absolute Ethanol	20	ml
Milli-Q water	80	ml

2. 70% iso-propanol + 0.1% Trifluoroacetic acid

Iso-propanol	70	ml
TFA	0.1	ml
Milli-Q water	30	ml

3. 50 mM Sodium phosphate buffer (pH 7.2)

Monosodium phosphate (Na_2HPO_4)	5.64	g
Disodium phosphate (NaH_2PO_4)	2.21	g
Milli-Q water	1	L

Dissolve two chemicals in 1 L of Milli-Q and adjust pH to 7.2

4. 50 mM Sodium phosphate buffer (pH 7.2) + 0.25, 0.5, 0.75 and 1.0 M NaCl

Prepare with the same ingredients with 50 mM sodium phosphate buffer and NaCl of 14.62 g, 29.25 g, 43.88 g and 58.5 g of NaCl to get the buffers containing 0.25, 0.50, 0.75, 1.0 M of NaCl, respective

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