SCREENING AND IDENTIFICATION OF PROBIOTIC LACTIC ACID BACTERIA FROM PLANT SAMPLES

Miss Ratthanatda Nuhwa

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Biotechnology
Common Course
Faculty of Science
Chulalongkorn University
Academic Year 2019

การคัดกรองและพิสูจน์เอกลักษณ์ของแบคทีเรียกรดแลคติกที่มีสมบัติเป็นโพรไบโอติกจากตัวอย่างพืช

น.ส.รัฐณัฏา หนูหว้า

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาเทคโนโลยีชีวภาพ ไม่สังกัดภาควิชา/เทียบเท่า
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2562

Thesis Title	SCREENING AND IDENTIFICATION OF PROBIOTIC LACTIC ACID BACTERIA FROM PLANT SAMPLES
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รัฐณัฏา หนูหว้า : การคัดกรองและพิสูจน์เอกลักษณ์ของแบคทีเรียกรดแลคติกที่มีสมบัติเป็นโพรไบโอติกจากตัวอย่างพืช
(SCREENING AND IDENTIFICATION OF PROBIOTIC LACTIC ACID BACTERIA FROM PLANT SAMPLES) อ.ที่ปรึกษาหลัก: ศ. ดร.อัญชริดา สวารชร, อ.ที่ปรึกษาร่วม: ศ. ดร.สมบูรณ์ ธนาศุภวัฒน์

การคัดแยกแบคทีเรียกรดแลกติกจำนวน 70 สายพันธุ์ จากตัวอย่างพืชได้แก่ ดอกไม้ 9 ตัวอย่าง (17 สายพันธุ์) เปลือกไม้ 3 ตัวอย่าง (7 สายพันธุ์) ผลไม้ 1 ตัวอย่าง (2 สายพันธุ์) ใบเมี่ยง 10 ตัวอย่าง (31 สายพันธุ์) และ ไซเลท 2 ตัวอย่าง (13 สายพันธุ์) จากผลการศึกษาลักษณะทางฟีโนไทป์และการวิเคราะห์ลำดับเบสช่วงยีน 16S rRNA สามารถพิสูจน์เอกลักษณ์ได้เป็น *Lactobacillus pentosus* (12 สายพันธุ์), *L. plantarum* subsp. *plantarum* (9 สายพันธุ์), *L. paracasei* subsp. *tolerans* (5 สายพันธุ์), *L. brevis* (1 สายพันธุ์), *L. silagincola* (1 สายพันธุ์), *L. kunkeei* (1 สายพันธุ์) และ *L. formosensis* (1 สายพันธุ์), *Enterococcus durans* (3 สายพันธุ์), *E. lactis* (2 สายพันธุ์), *E. faecalis* (1 สายพันธุ์), *E. faecium* (1 สายพันธุ์), *E. gallinarum* (1 สายพันธุ์) และ *E. gilvus* (1 สายพันธุ์), *Pediococcus acidilactici* (1 สายพันธุ์), *P. pentosaceus* (1 สายพันธุ์) และ *Aerococcus urinaeequi* (1 สายพันธุ์) ในการศึกษานี้พบว่าสายพันธุ์ FM11-1^{T} ที่แยกจากดอกมะเขือพวง (*Solanum torvum*) เป็นแบคทีเรียสปีชีส์ใหม่ที่มีความใกล้เคียงกับ *E. faecium* NRIC 1145^{T} (98.79 %), *E. durans* NBRC 100479^{T} (98.72 %), *E. lactis* LMG 25958^{T} (98.49 %) และ *E. ratti* DSM 15687^{T} (98.02 %) จากผลวิเคราะห์ความคล้ายคลึงของลำดับเบสช่วงยีน 16S rRNA และจีโนม รวมถึงแผนภูมิวิวัฒนาการ จึงเสนอตั้งชื่อว่า *Enterococcus solani* จากการคัดกรองการลดคลอเลสเตอรอลโดยการสร้างเอนไซน์ไบล์ซอลท์ไฮโดรเลสและการนำคอเลสเตอรอลไปใช้ ฤทธิ์ต้านจุลชีพ และ ความเป็นพิษต่อเซลล์มะเร็ง ของแบคทีเรียกรดแลกติกที่แยกได้ พบว่ามี 28 สายพันธุ์ สามารถสร้างเอนไซน์ไบล์ซอลท์ไฮโดรเลส 19 สายพันธุ์ สามารถนำคอเลสเตอรอลไปใช้มากกว่า 45 % โดยพบว่าสายพันธุ์ CRM44-2 สามารถนำคอเลสเตอรอลไปใช้ได้สูงที่สุดถึง 85.5% นอกจากนี้พบว่ามี 8 สายพันธุ์ที่มีสร้างเอนไซน์ไบล์ซอลท์ไฮโดรเลส และ สามารถนำคอเลสเตอรอลไปใช้ได้มากว่า 45% ได้แก่ FM3-1, FM11-1, FM11-2, CM28-3, CM38-1, CRM41-1, CRM55-2 และ NWM60-2 พบว่าสายพันธุ์ CRM51-2 มีฤทธิ์ต้านเชื้อ *Bacillus subtilis* ATCC 6633 ส่วนสายพันธุ์ CM28-3 และ CRM42-1 มีฤทธิ์ความเป็นพิษต่อเซลล์มะเร็งลำไส้ใหญ่ (Caco-2 cell) แต่ไม่มีความเป็นพิษต่อเซลล์ปกติ (Vero cells) และ เซลล์มะเร็งตับ (HepG2 cells) เมื่อศึกษาคุณสมบัติทั่วไปของการเป็นโพรไบโอติกของแบคทีเรียกรดแลคติก 31 สายพันธุ์ที่มีฤทธิ์เบื้องต้นพบว่าทั้ง 31 สายพันธุ์ทนต่อสภาวะพีเอช 3 และทนกรดน้ำดี 0.3 % นาน 3 ชั่วโมง และ มี 13 สายพันธุ์ที่สามารถในการเกาะติดบนเซลล์ Caco-2 cell โดยพบว่า *L.brevis* CM38-1 สามารถสร้างเอนไซน์ไบล์ซอลท์ไฮโดรเลส และ นำคอเลสเตอรอลไปใช้ได้ถึง 62.5% และ สามารถยึดเกาะกับผนังลำไส้ได้ดีที่สุดถึง 6.0 + 1.0 % นอกจากนี้ *L. plantarum* subsp. *plantarum* CM28-3 สามารถสร้างเอนไซน์ไบล์ซอลท์ไฮโดรเลส และ นำคอเลสเตอรอลไปใช้ได้ถึง 46.5% สามารถยึดเกาะกับผนังลำไส้ได้ดีถึง 0.7 + 1.0 % และมีฤทธิ์ความเป็นพิษต่อเซลล์มะเร็งลำไส้ใหญ่ (Caco-2 cell) ได้ถึง 64.0 + 0.1 % ดังนั้นจึงเป็นสายพันธุ์ที่มีคุณสมบัติเป็นโปรไบโอติกได้ดีกว่าเมื่อเทียบกับ *L. rhamnosus GG* ที่เป็นสายพันธุ์ควมคุม

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต .....
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ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

5972114023 : MAJOR BIOTECHNOLOGY

KEYWORD: Lactic acid bacteria; Probiotic; Plant sample

Ratthanatda Nuhwa :

SCREENING AND IDENTIFICATION OF PROBIOTIC LACTIC ACID BACTERIA FROM PLANTSAMPLES.

Advisor: Prof. ANCHARIDA SAVARAJARA, D.Eng. Co-advisor: Prof. SOMBOON TANASUPAWAT, Ph.D.

Seventy lactic acid bacteria (LAB) were isolated from flowers (17 strains), 3 tree barks (7 strains), one fruit (2 strains), ten fermented tea leaves (31 strains) and two silage (13 strains). They were identified as *Lactobacillus pentosus* (12 strains), *L. plantarum* subsp. *plantarum* (9 strains), *L. paracasei* subsp. *tolerans* (5 strains), *L. brevis* (1 strain), *L. silagincola* (1 strain), *L. kunkeei* (1 strain), and *L. formosensis* (1 strain), *Enterococcus durans* (3 strains), *E. lactis* (2 strains), *E. faecalis* (1 strain), *E. faecium* (1 strain), *E. gallinarum* (1 strain) and *E. gilvus* (1 strain), *Pediococcus acidilactici* (1 strain) and *P. pentosaceus* (1 strain), and *Aerococcus urinaeequi* (1 strain) based on their phenotypic characteristics and 16S rRNA gene sequences. Strain FM11-1^T, a novel species isolated from the flower of *Solanum torvum*, was closely related to *E. faecium* NRIC 1145^T (98.79 %), *E. durans* NBRC 100479^T (98.72 %), *E. lactis* LMG 25958^T (98.49 %), and *E. ratti* DSM 15687^T (98.02 %) based on the 16S rRNA gene sequence, genome sequence, and phylogenetic tree. Therefore, it was proposed as *Enterococcus solani* sp. nov. The screening of bile salt hydrolase, cholesterol assimilation, antimicrobial activity, and cytotoxicity against Caco-2 cells revealed that 28 strains showed bile salt hydrolase activity while 19 strains exhibited cholesterol assimilation more than 45 %. Strain CRM44-2 showed the highest cholesterol assimilation of 85.5 %. Furthermore, the strains FM3-1, FM11-1, FM11-2, CM28-3, CM38-1, CRM41-1, CRM55-2, and NWM60-2 exhibited bile salt hydrolase activity and cholesterol assimilation more than 45 %. Strain CRM51-2 showed antimicrobial activity against *Bacillus subtilis* ATCC 6633. Strains CM28-3 and CRM42-1 showed cytotoxic effects against colorectal cancer cell lines (Caco-2 cells) with non-toxicity to Vero cells. All isolates showed no cytotoxic effects against Vero and HepG2 cells. For the probiotic properties, 31 strains showed pH 3.0 and 0.3 % of bile acid tolerance for 3 hours by decreasing 2 log cycles while 13 strains showed the adhesion ability. *L. brevis* strain CM38-1 isolated from fermented tea leaves, showed bile salt hydrolase activity, cholesterol assimilation of 62.5 % and adhesion ability of 6.0 + 1.0 % as the candidate probiotics with cholesterol-lowering. Moreover, *L. plantarum* subsp. *plantarum* strain CM28-3 isolated from fermented tea leaves, showed bile salt hydrolase activity, cholesterol assimilation of 46.5 %, cytotoxicity against Caco-2 cells of 64.0 + 0.1 %, and adhesion ability of 0.7 + 1.0 %. Therefore, it is suggested to be the candidate probiotics with higher cholesterol-lowering activity compared to *L. rhamnosus* GG as positive control.

Field of Study:	Biotechnology	Student's Signature .....
Academic Year:	2019	Advisor's Signature .....
		Co-advisor's Signature .....

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to my thesis advisor, Professor Dr. Ancharida Savarajara, who gave me the excellent chance, guidance, and acceptance to be a student under her consideration, throughout this study.

My sincere thanks are expressed to my co-advisors, Professor Dr. Somboon Tanasupawat, for his valuable support and expected guidance throughout this research and I would also like to extend my gratitude to Assistant Professor Dr. Malai Taweechotipatr, for her kindly bits of advice and valuable support about the part of cytotoxicity and probiotic properties. Furthermore, the following persons are also greatly acknowledged;

Dr. Jaruwan Sitdhipol and all staff, Biodiversity Research Centre, Thailand Institute of Scientific and Technological Research (TISTR) for special support and kindly suggestion throughout this study.

Assistant Professor Dr. Kobchai Pattaragulwanit, Associate Professor Dr. Suchada Chanprateep Napathorn and Dr. Thanyanuch Kriangkripipat, for serving as the committee and for their editorial assistance and comments.

I would like to thank all staffs and friends of Program in Biotechnology, Faculty of Sciences, Chulalongkorn University; the Institute of Biotechnology and Genetic Engineering, Chulalongkorn University; Department of Biochemistry and Microbiology and the Pharmaceutical Research Instrument Center, Faculty of Pharmaceutical Sciences, Chulalongkorn University and also Department of Microbiology, Faculty of Medicine, Srinakharinwirot University for teaching me, their kindness helpful, support, and supplying instruments throughout the research study.

Finally, I am extremely grateful to thank my family for their love, understanding, encouragement, and unconditional support throughout my education.

Ratthanatda Nuhwa

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CHAPTER I

INTRODUCTION

Lactic acid bacteria (LAB) are Gram-positive, cocci or bacilli, catalase-negative, non-motile, non-sporulating, and aerobic or facultative anaerobic or strictly anaerobic bacteria that can mostly produce lactic acid from glucose. They are classified based on production of lactic acid from various fermentable carbohydrate, and 16S rRNA gene sequence into several genera as *Lactobacillus, Lactococcus, Enterococcus, Pediococcus, Leuconostoc, Streptococcus, Lactovum, Oenococcus, Carnobacterium, Aerococcus, Tetragenococcus, Vagococcus, Paralactobacillus*, *Fructobacillus, Weissela,* etc (Pot *et al.*, 2014). They are commonly distributed in plants, decomposing plants, fermented foods, soil, dairy products, and in digestive systems of human and animals as normal flora (Maeda *et al.*, 2014).

LAB are well-known 'probiotics' that have the potential benefits for host health and nutritional advantage. Food and Agricultural Organization of the United Nations/World Health Organization (FAO/WHO) definite that 'The probiotics are live microorganisms which when administered in adequate amounts confer a beneficial health effect on the host' (Joint, 2002). *Lactobacillus*, *Bifidobacterium*, *Streptococcus*, *Pediococcus,* and *Enterococcus* are usually genera used as probiotics for humans and animals that can resist acid and bile salt, adhere to intestinal tissues, and colonize in the gastrointestinal tract of host (Tomé *et al.,* 2008).

Nowadays, LAB are used one or mix strains as the component in commercial products, particularly *Lactobacillus* used in dairy products. They can provide relief for lactose intolerant, traveling diarrhea, and infective diarrhea. Other evidence benefits of LAB such as remodeling of microbial communities in digestive system, suppression of pathogens, stimulation of the immune system by up-regulation of anti-inflammatory factors, suppression of pro-inflammatory factors, suppression of established remaining cancer, and cholesterol-lowering (Thamacharoensuk *et al.*, 2017).

Many kinds of research have been investigated the cholesterol-lowering effect of LAB by *in vitro* and *in vivo* studies after discovering the hypocholesterolemic effect of the fermented milk containing *Lactobacillus* strain in humans. LAB strains with the cholesterol-lowering effect such as *Lactobacillus acidophilus, L. casei, L. paracasei subsp. paracasei, L. fermentum, L. rhamnosus, L. plantarum,* and *Enterococcus faecium* have been reported M. Kumar *et al.*, 2012. The cholesterol-lowering mechanisms of LAB have been proposed to the direct mechanism as assimilation of cholesterol during the LAB growth and the indirect mechanism as suppression of bile acid reabsorption mediated by bile salt hydrolase (BSH) (Ahn *et al.*, 2003; Begley *et al.*, 2006). Therefore, isolation and identification of lactic acid bacteria from plant and fermented plant samples to find the new species or specific strains showing cholesterol lowering, antimicrobial activity, or cytotoxicity with their probiotic properties are objectives in this research.

The aim of this study was screening of probiotic lactic acid bacteria with cholesterol lowering, antimicrobial activity, and cytotoxicity against cancer cells.

The main objectives of this research are as follows:

1. To isolate LAB from plant samples and identify based on phenotypic characteristics and 16S rRNA gene sequence analysis.

2. To screen LAB that produces bile salt hydrolase or reduce cholesterol level *in vitro* as assimilate cholesterol, antimicrobial activities, and cytotoxicity against cancer cells.

3. To determine the selected LAB that possess probiotic properties such as the acid and bile tolerance and adherence property

CHAPTER II

LITERATURE REVIEW

2.1 Definition, diversity, and application of lactic acid bacteria

Lactic acid bacteria (LAB) are a group of gram-positive, cocci or bacilli, catalase-negative, non-motile, usually non-spore forming, and aerobic or facultative anaerobic or strictly anaerobic bacteria that produce lactic acid as the end-product during glucose fermentation. They are defined to differentiate from other microorganisms by their ability to ferment glucose to lactic acid, hence the name as lactic acid bacteria (LAB).

LAB are divided into the large group of genera belonged to the order *Lactobacillales* of phylum *Firmicutes* as *Aerococcus, Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Oenococcus, Pediococcus, Streptococcus, Tetragenococcus, Vagococcus, and Weissela* based on molecular characteristics as shown the phylogenetic tree of LAB in Figure 2.1. Nowadays, LAB are classified based on Gram's stain, production of lactic acid from various fermentable carbohydrates, and 16S rRNA gene sequence into the genera of six families such as *Aerococcus, Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Lactovum, Leuconostoc, Oenococcus, Pediococcus, Streptococcus, Symbiobacterium, Tetragenococcus, Vagococcus, Paralactobacillus, Fructobacillus, Weissela* while *Bifidobacterium, Parascardovia, Scardovia, Bacillus, Geobacillus*, *Halobacillus, Halolactibacillus*,

Paraliobacillus, and *Sporolactobacillus* as the physiologically related genera of LAB (Pot *et al.*, 2014).

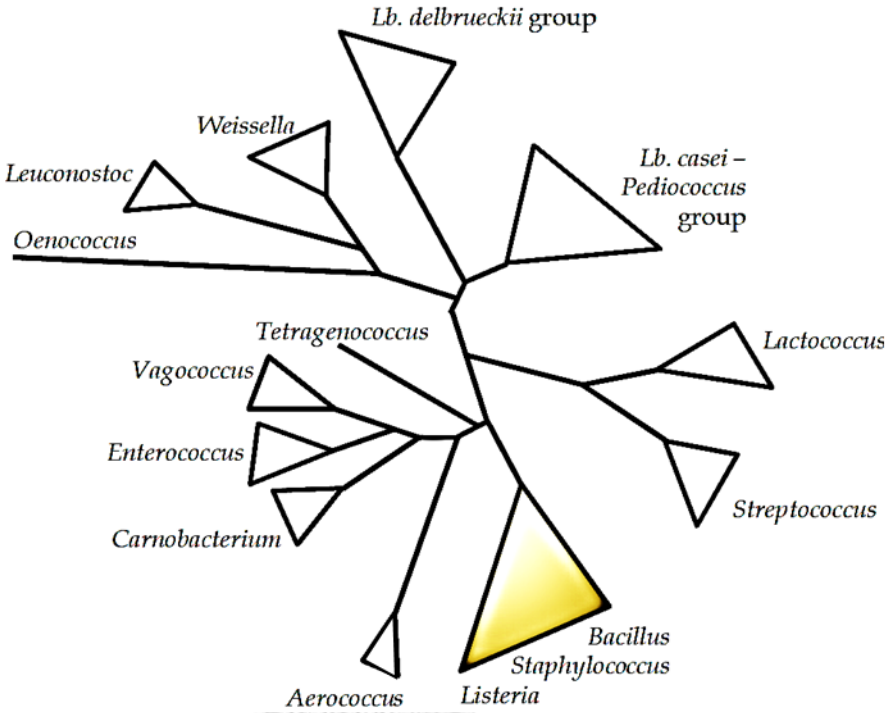

Figure 2.1 Unrooted phylogenetic tree showed the estimated evolutionary distances of lactic acid bacteria (Axelsson, 2004)

The colony of LAB are small as in size 2–3 mm. The G+C content in their chromosomal DNA are less than 50 mol%. Typically, they are mesophilic bacteria that can grow at temperature ranges from 5 to 45 °C, pH ranges from 5.5 to 6.5, and tolerate to acidic and alkaline conditions. They can grow under aerobic and anaerobic environments. However, the differential characteristics among genera of LAB are shown in Table 2.1.

Table 2.1 Differential characteristics between genera of LAB (Lahtinen *et al.*, 2011)

Genera	Characteristics								
	Cell shape	CO_2 from Glucose	Growth at 10°C	Growth at 45°C	Growth in 6.5% NaCl	Growth in 18% NaCl	Growth at pH 4.4	Growth at pH 9.6	Lactic acid isomer
Aerococcus	Cocci	-	+	-	+	-	-	+	L
Carnobacterium	Rods	-	+	-	ND	-	ND	-	L
Enterococcus	Cocci	-	+	+	+	-	+	+	L
Tetrageonococcus	Rods			-	+	+	Variable	+	L
Vagococcus	Cocci		+	-	-	-	ND	-	ND
Lactobacillus	Rods	Variable	Variable	Variable	Variable	-	Variable	-	L, DL
Pediococcus	Tetrads	-	Variable	Variable	Variable	-	+	-	D, L, DL
Leuconostoc	Cocci	+	+	-	Variable	-	Variable	-	D
Oenococcus	Cocci	+	+	-	Variable	-	Variable	-	D
Weissella	Cocci	+	+	-	Variable	-	Variable	-	D, DL
Lactococcus	Cocci	-	+	-	-	-	Variable	-	L
Streptococcus	Cocci	-	-	Variable	-	-	-	-	L

ND, not determined, +, Positive, -, Negative

Genus *Lactobacillus*

The type species of this genus is *Lactobacillus delbrueckii* (Jlavescens, 1983). They are Gram-positive, facultative anaerobic or microaerophilic, rod-shaped, non-spore-forming bacteria that produced lactic acid as a major part of the lactic acid bacteria (LAB). The differentiated characteristics between the genus *Lactobacillus* and the other genera of LAB are shown in Table 2.1. At the time of writing, this genus comprises 241 species and 29 subspecies with validly published names in the *List of Prokaryotic names* (www.bacterio.net/lactobacillus.html). Recently, new *Lactobacillus* were isolated from plant samples and were reported such as *L. musae* isolated from banana fruits (Chen *et al.*, 2017) and *L. pentosiphilus* isolated from silage fermented with orchard grass silage, corn silage, soybean curd residue, hay, and compound feed (Tohno *et al.*, 2017).

Genus *Enterococcus*

The type species of this genus is *Enterococcus faecalis* (Schleifer and Kilpper-Bälz, 1984). They are Gram-positive cocci, facultative anaerobic, catalase-negative, diplococci or short chains, non-spore-forming bacteria that produced lactic acid as other major member of the lactic acid bacteria (LAB). The different characteristics between the genus *Enterococcus* and the other genera of LAB are shown in Table 2.1. At the time of writing, this genus comprises 58 species and 2 subspecies with validly published names in the *List of Prokaryotic names* (www.bacterio.net/enterococcus.html). Recently, new *Enterococcus* was isolated

from plant samples and were reported such as *E. xiangfangensis* isolated from Chinese pickle (Li *et al.*, 2014).

Genus *Pediococcus*

The type species of this genus is *Pediococcus damnosus* (Skerman *et al.*, 1989). They are Gram-positive, facultative anaerobic or microaerophilic, rod-shaped in pairs or tetrads, non-spore-forming bacteria that produced lactic acid as lactic acid bacteria (LAB). The differentiated characteristics between the genus *Pediococcus* and the other genera of LAB are shown in Table 2.1. At the time of writing, this genus comprises 15 species with validly published names in the *List of Prokaryotic names* (www.bacterio.net/pediococcus.html).

Genus *Aerococcus*

The type species of this genus is *Aerococcus viridans* (Williams *et al.*, 1953). They are Gram-positive cocci, facultative anaerobic, catalase-negative, arrangement in pairs (diplococci) or short chains, non-spore-forming bacteria**,** produced lactic acid that are human pathogens. The differentiated characteristics between the genus *Enterococcus* and the other genera of LAB are shown in Table 2.1. At the time of writing, this genus comprises 8 species with validly published names in the *List of Prokaryotic names* (www.bacterio.net/aerococcus.html).

LAB are divided into two groups based on the metabolic pathways for oxidizing carbohydrate, as homo- or heterofermentative LAB. Homofermentative LAB can convert glucose to lactic acid (more than 80%) through the Embden-Meyerhof-Parnas (EMP) or glycolysis pathway while the other convert glucose to lactic acid (50%), ethanol, acetic acid, and CO_2 through the phosphoketolase pathway as shown in Figure 2.2 (Reddy *et al.*, 2008; Hayek and Ibrahim, 2013). They are heterotrophic that have limited biosynthetic ability, requiring a complex nutrition. Moreover, they can produce many products such as mainly organic acids, alcohol and CO_2, including aromatic molecules, vitamins and bioactive peptides such as bacteriocins (Trias, 2008).

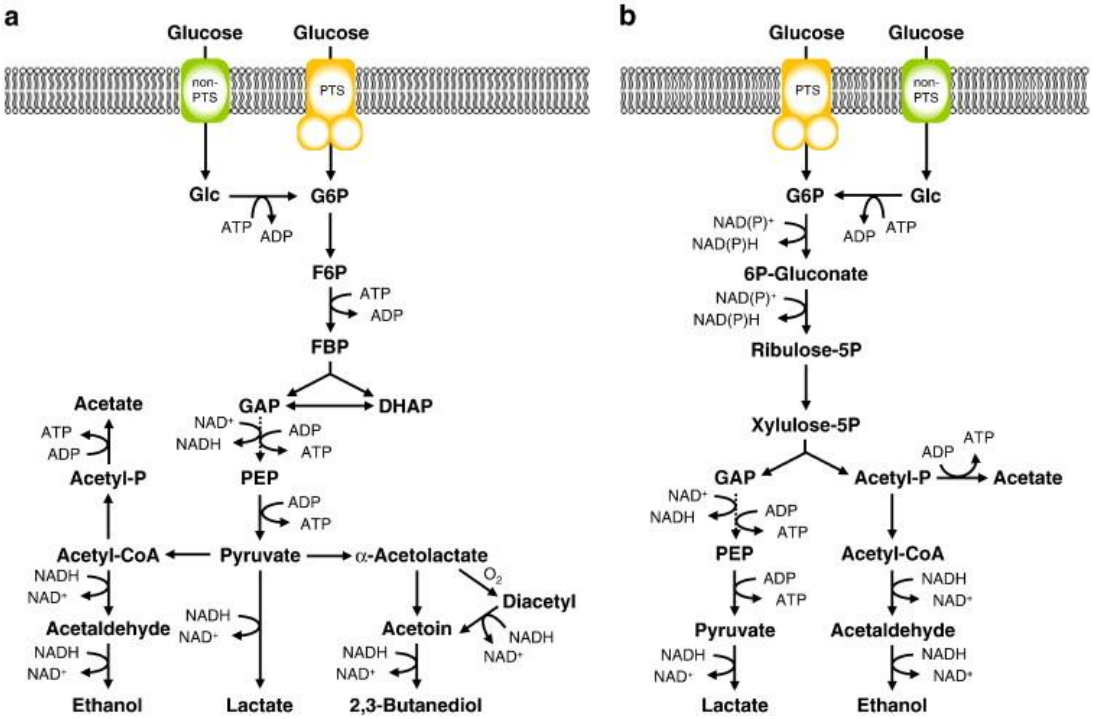

Figure 2.2 Glucose metabolic pathways of two lactic acid bacteria groups as (A) Homofermentative LAB, (B) Heterofermentative LAB (Gaspar *et al.*, 2013)

LAB distribute in wide environments with high carbohydrates, such as dairy products, beverages, fermented meats, fermented plants, vegetables, fruits, including gastrointestinal tract, oral cavity, and vagina of animals and human as shown in Table 2.2. Many LAB have been found in plants especially on surface, flower, and fruit, such as *Lactobacillus. plantarum, L. brevis, L. coryniformis, L. paracasei, L. curvatus, L. sakei, L. fermentum, Enterococcus faecalis, E. faecium, E. Hirae, E. mundtii, E. casseliflavus, E. Sulfureus, Leuconostoc mesenteroides* subsp. *mesenteroides, Leuc mesenteroides* subsp. *dextranicum, Leuc fallax, Leuc citreum, Leuc gelidum,* and *Leuc kimchi* (Hammes and Hertel, 2009). They play an important role in fermented products such as silage, kimchi, pickles, beer, wine, fermented milk, and fermented meats. Moreover, lactic acid produced from LAB is applied in a wide variety of industrial applications such as preservatives or flavor additives in food and beverage industries and as precursors of the polylactic acid (PLA) production in pharmaceutical, leather, textile, and biodegradable plastic industries as well as chemical feed stock. Lactic acid fermentation by LAB can be found in optically stereoisomer L-, D- and DL-racemic mixture of lactic acid. The isomer L-lactic acid is generally used in food and pharmaceutical industries because humans have L-lactate dehydrogenase so particularly metabolizing only L-lactic acid form. For medical applications, lactic acid polymers formed L- and D-form (PLA) can be used as sutures, orthopedic implants, and drug delivery systems (Reddy *et al.*, 2008; Gaspar *et al.*, 2013).

Food grade LAB are regulated in different ways in the different countries. The USA Federal Food, Drug and Cosmetic Act considered LAB as the GRAS (generally recognized as safe), defined as 'generally recognized, among experts qualified by scientific training end-experience to evaluate its safety, as having been adequately shown through scientific procedures to be safe under the conditions of its intended use'. The European Food Safety Authority (EFSA) has proposed the listed microorganisms with the safety use based on the list of Qualified Presumption of Safety (QPS), defined as 'a belief or assumption based on reasonable evidence', recommends the use of LAB in foods whether as traditional starters, food supplements, or probiotics. This list contained over 30 species of LAB such as *Lactobacillus*, *Lactococcus lactis, Leuconostoc citreun, Leuc lactis, Leuc mesenteroides, Pediococcus acidilactici, P. pentosaceus, P. dextrinicus, Streptococcus thermophilus*, etc (Wessels *et al.*, 2004; EFSA Panel on Biological Ricci *et al.*, 2017). However, enterococci and some lactobacilli as *L. casei* and *L. rhamnosus* have been documented in the opportunistic infections that associated with the pathogenic strains (Vesterlund *et al.*, 2007). To date, the genus *Enterococcus* has not yet obtained the GRAS but *E. faecium* and *E. faecalis* are used as probiotics and feed additives to prevent diarrhea or improve growth in animals, while some members were elucidated for the probiotic potential such as *E. munditii, E. durans*, and *E. hirae* (Hanchi *et al.*, 2018).

Most genera of LAB are bacteria with the lowest risk to human health that are extensively used in food and industrial fermentations around the world for many years and are associated with the health-promoting probiotics as shown in Figure 2.3. Several studies revealed that the benefit of LAB on human health as 'probiotics' are antimicrobial activity against gastrointestinal infections, lactose metabolism improvement, antimutagenicity, anti-carcinogenicity, serum cholesterol lowering, anti-inflammatory, and immune system stimulation (Shah, 2007). The antimicrobial mechanisms of LAB are due to the combined action of several metabolites produced during their fermentations, especially low pH environment by organic acids (lactic, acetic, and propionic acids) as inhibiting or modifying the active transport processes, reactions, and membrane potential of the microbiota, no catalase as accumulating hydrogen peroxide (H_2O_2) which is toxic for other microorganisms, CO_2 from heterolactic fermentation create the anaerobic and low pH microenvironment which is toxic for some aerobic bacteria, and some LAB can produce antimicrobial peptides as bacteriocins (Trias Mansilla, 2008).

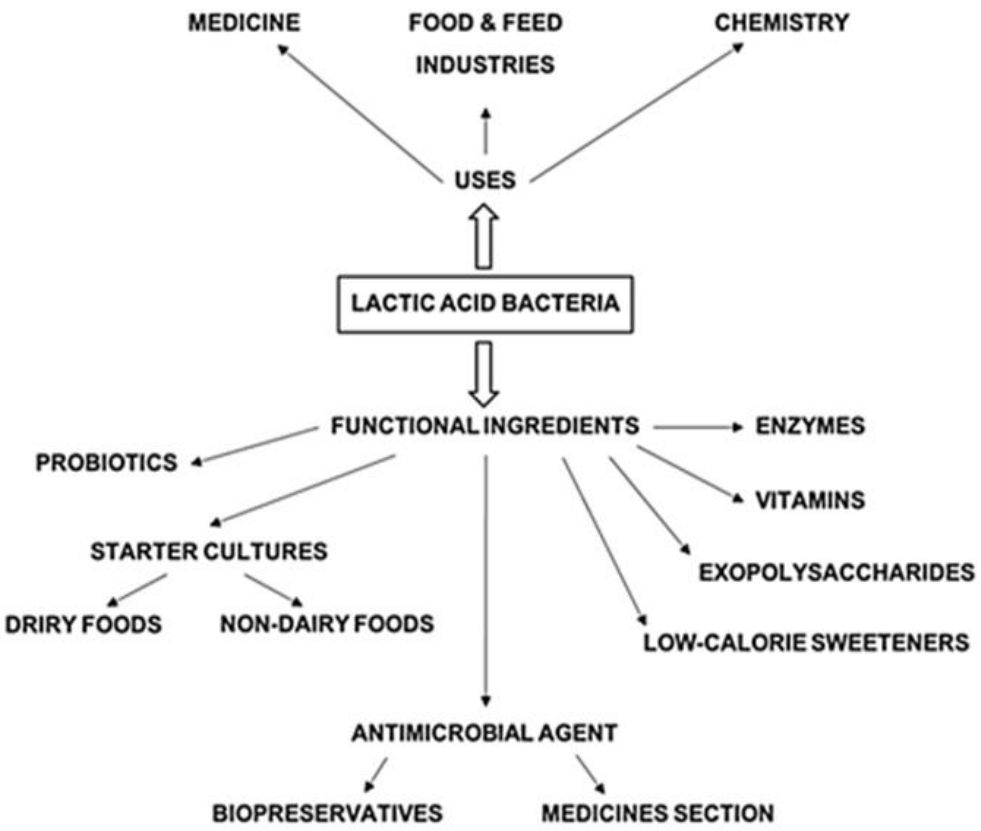

Figure 2.3 The benefits of lactic acid bacteria (Florou-Paneri *et al*., 2013).

Table 2.2 Distribution of lactic acid bacteria from human, animals and foods.

Habitat	Source	Species	References
GI tract	Human	*L. ruminis, L. crispatus, L. gasseri, L. paracasei, L. acidophilus, L. plantarum, L. delbrueckii*	Vaughan *et al.*, 2005
	Cattle	*S. bovis*, *W. paramesenteroides, L. plantarum*, *L. reuteri, L. mucosae*, *Lc. lactis* subsp *lactis, E. hirae*, *E. faecium*, *E. mundtii*	Espeche *et al.*, 2009
Oral cavity	Human	*L. gasseri, L. vaginalis, S. salivarius, S. mitis, L. paracasei, S. intermedius, S. oralis, S. mutans, S. gordonii*	Dal Bello and Hertel, 2006; Aas *et al.*, 2008
Vagina	Human	*L. crispatus, L. fermentum, L. salivarius, L. gasseri, L. jensenii, L. reuteri, L. vaginalis, P. acidilactici*	L. Jin *et al.*, 2007
Fermented meat	Sausage	*L. plantarum, L. sakei, L. rhamnosus, L. brevis, L. curvatus, W. cibaria*	Drosinos *et al.*, 2007; Pringsulaka *et al.*, 2012
Fermented fish	Pla-som	*Lc. garvieae, S. bovis, W. cibaria, L. plantarum, L. fermentum, P. pentosaceus*	Kopermsub and Yunchalard, 2010
Dairy products	Fermented milk	*L. helveticus, L. kefiranofaciens, L. delbrueckii* subsp. *bulgaricus, S. thermophiles*	Watanabe *et al.*, 2008; Thirabunyanon *et al.*, 2009
	Yoghurt	*S. thermophilus, L. acidophilus, L. delbrueckii* subsp. *bulgaricus*	Gaus *et al.*, 2006
	Cheese	*L. paracasei, L. plantarum, Lc. lactis* subsp. *lactis, E. faecalis, Leuc. pseudomesenteroides, Leuc. mesenteroides*	González *et al.*, 2007; Nikolic *et al.*, 2008

Table 2.2 Distribution of lactic acid bacteria from human, animals and foods. (continued)

Habitat	Source	Species	References
Beverages	Wine	*L. buchneri, L. hilgardii, L. plantarum, L. casei, L. paracasei, L. collinoides, L. brevis, L. mali, Leuc mesenteroides, P. pentosaceus, O. oeni, P. parvulus*	J.M. Landete *et al.*, 2005; J. Jin *et al.*, 2008
	Beer	*L. brevis, L. lindneri, L. delbrueckii* subsp. *delbrueckii, L. delbrueckii* subsp. *bulgaricus, P. damnosus, P. acidilactici*	Suzuki *et al.*, 2005; Sawadogo-Lingani *et al.*, 2007
Silage	Corn stover	*L. plantarum, L. pentosus, L. brevis, W. cibaria, Leuc lactis, E. mundtii*	Pang *et al.*, 2011
	Grass	*E. gallinarum, L. acidipiscis, L. curvatus, L. coryniformis, Lc. garvieae, Leuc. pseudomesenteroides, Lc. lactis* subsp. *cremoris, P. acidilactici, P. pentosaceus*	Tohno *et al.*, 2012; Santos *et al.*, 2013
Fermented vegetable	Kimchi	*L. acidophilus, L. casei, L. plantarum, L. sakei, Leuc mesenteroides*	J.-H. Chang *et al.*, 2010
	Fermented olives	*Leuc mesenteroides, L. paraplantarum, L. plantarum, L. pentosus, L. paracasei* subsp. *paracasei*	Argyri *et al.*, 2013
Plant	Fruit	*Leuc. pseudomesenteroides, L. agilis, L. bifermentans, L. plantarum, L. hilgardii, L. fructosus, L. fermentum, L.brevis, W. minor, Streptococcus* spp.	Nyanga *et al.*, 2007; Ruiz Rodriguez *et al.*, 2019
	Flower	*W. cibaria, Leuc. pseudomesenteroides, L. brevis, Leuc. mesenteroides* subsp. *mesenteroides, Lc. lactis, Lc. lactis* subsp. *lactis, E. faecalis, E. casseliflavus, E. gallinarum*	

2.2 Lactic acid bacteria as probiotics

In 2002, Food and Agricultural Organization of the United Nations/World Health Organization (FAO/WHO) defined the probiotics as 'The live microorganisms, which when administered in adequate amounts, confer a health benefit on the host' and recommend that LAB are the generally recognized as safe (GRAS), the safety of the potential probiotic should be assessed by the minimum required tests (Shah, 2007; Saad *et al.*, 2013). In Europe, EFSA (European Food Safety Authority) recommended the safety consumption of microorganisms based on the QPS guideline (Qualified Presumption of Safety). Many LAB are generally used as 'probiotics', including the genera *Lactobacillus, Bifidobacterium, Streptococcus, Lactococcus, Enterococcus, Pediococcus,* and *Leuconostoc* as shown in Table 2.3.

Probiotic properties of LAB as the generally recognized as safe (GRAS) status should be survived and tolerated through the acidic and high protease condition in stomach and the alkaline bile salt condition in small intestine. Moreover, epithelial adhesion of probiotics is a desirable property for living and colonizing in the gastrointestinal tract. Criteria for selection of probiotic bacteria are shown in Table 2.4. Probiotic microorganisms should also be technologically suitable for production and should survive during industrial manufacturing processes. The important criteria for probiotic use generally based on specific activity in nutritional or health benefit, viability and safety. According to safety, acceptable criteria include non-pathogenic, non-hemolytic, antibiotic resistance, generally recognized as safe (GRAS)

microorganism as should be normal inhabitant for the target purposes such as human origin or generally fermented foods for human probiotics. Other parameters are health beneficial activities based on *in vitro* and *in vivo* tests, including antimicrobial activity, immune response stimulation, cholesterol lowering, lactase or other enzyme activities, and vitamin production (Gaggìa *et al.*, 2010; Linares *et al.*, 2017).

Nowadays, the commercial probiotics are mainly LAB with over one hundred species recognized such as *L. acidophilus, L. rhamnosus, L.reuteri, L. plantarum, L. casei, L. bulgaricus, L. delbrueckii, L. helveticus*. They are used in commercial products as one or combination of LAB strains, commonly in the fermented dairy products and the pills as capsules or microencapsulated forms. In decades, *Lactobacillus* and *Bifidobacterium* have been greatly interested and investigated with beneficial effect on human health because they isolated from human intestinal normal flora as GRAS. Recently studies, many advantages of lactic acid bacteria (LAB) and bifidobacteria have been applied genetic engineering for promising carrier microorganisms as DNA microbial biosensor vehicles (J. M. Landete and Arqués, 2017).

Table 2.3 Microorganisms used as probiotics (Saad *et al*., 2013; Kailasapathy, 2013)

Group of probiotics	Species
Lactobacillus	*L. acidophilus*, L. amylovorus*, L. brevis*, L. casei*, L. crispatus*, L. delbreuckii subspecies bulgaricus, L. fermentum*, L. gallinarum*, L. gasseri, L. helveticus, L. johnsonii, L. plantarum*, L. paracasei, L. reuteri*, L. rhamnosus**
Other LAB	*Enterococcus faecalis*, E. faecium*, Lactococcus lactis*, Leuconstoc mesenteroides*, Pediococcus acidilactici*, P. pentosaceus*, Streptococcus thermophilus, S. cremoris, S. diacetylactis, S. intermedius, S. salivarius*
Bifidobacterium	*B. adolescentis, B. animalis*, B. bifidum, B. breve, B. essensis, B. infantis, B. lactis*, B. laterosporus, B. longum*, B. thermophilum**
Other non-LAB	*Bacillus cereus* var. *toyoi*, Escherichia coli* strain *nissle, Saccharomyces cerevisiae*, Saccharomyces boulardii, Propionibacterium freudeneichii**

* Commonly application in animals

Table 2.4 Expected criteria of probiotics (Gaggìa *et al.*, 2010)

Appropriateness	
I	Accurate taxonomic identification
II	Normal inhabitant of the species targeted
III	Nontoxic, nonpathogenic
Technological suitability	
IV	Amenable to mass production and storage: adequate growth, recovery, concentrations, freezing, dehydration, storage, and distribution
V	Viability at high populations (preferred at 10^6 –10^8 CFU)
VI	Stability of desired characteristics during culture preparation, storage, and delivery
VII	Provides desirable organoleptic qualities (or no undesirable qualities)
VIII	Genetically stable
IX	Genetically amenable
Competitiveness	
X	Survival, colonization and being metabolically active in the targeted site
XI	Resistant to bile
XII	Resistant to acid
XIII	Competition with the resident microbiota
XIV	Adhesion to epithelium or mucus
Performance and functionality	
XV	Ability to exert at least one scientifically supported health-promoting properties
XVI	Antagonistic toward pathogenic bacteria
XVII	Production of antimicrobial substances
XVIII	Modulation of immune responses
XIX	Antimutagenic
XX	Anticarcinogenic
XXI	Production of bioactive compounds

2.3 Functionality of probiotic lactic acid bacteria

The most common microorganisms used as probiotics are lactic acid bacteria and *bifidobacteria*, even though other bacteria and certain yeasts are also used. The two genera, *Lactobacillus* and *Bifidobacterium*, are non-pathogenic, resist to gastric acid, bile salts, pancreatic enzymes, and technological processes. They have revealed to effectively adhere the epithelial tissue, colonize in the gastrointestinal, and produce antimicrobial substances. Many probiotics usually used in animals include *Lactobacillus, Bacillus, Pediococcus, Enterococcus,* and yeasts to improve animal health, promote growth rates, and increase production of milk or eggs (Bernardeau and Vernoux, 2013). For functional properties of probiotics in human, they have been investigated in various applications such as potential health benefits, clinical prevention, and therapeutic uses.

Lactic acid bacteria (LAB) can convert hexose sugars to lactic acid thus creating a hostile acid environment that inhibits the growth of several harmful bacteria and spoilage microorganisms. They have received tremendous attention due to the health-promoting properties as probiotics that their claimed health benefits are shown in Table 2.5.

Table 2.5 The published health benefit claims of LAB as probiotics (Fijan, 2014)

Species of LAB	Published health claims
Lactobacillus rhamnosus	Reduction of viral-associated pulmonary damage; prevention and reduction of severe atopic dermatitis in children; reduction of the developing allergic disease risk; anti-diabetic potential; prevention of necrotizing enterocolitis in newborns; prevention or treatment of bacterial vaginosis; aid in weight loss of obese; treatment of acute gastroenteritis in children; reduction of the rhinovirus infection risk in preterm infants; protection of human colonic muscle from lipopolysaccharide-induced damage
L. acidophilus	Treatment of travellers' diarrhoea; reduction of hospital staying of children with acute diarrhoea; antifungal activity; prevention or treatment of bacterial vaginosis; treatment of *C. difficile*-associated diarrhoea; reduction of incidence of febrile urinary tract infections in children; reduction of irritable bowel syndrome symptoms
L. plantarum	Prevention of endotoxin production; antifungal activity; reduction of irritable bowel syndrome symptoms
L. brevis	Protective role in bile salt tolerance; reduction in plague acidogenicity
L. fermentum	Prevention or treatment of bacterial vaginosis; blockage of adherence of pathogenic microorganisms on vaginal epithelium; potential for reduction of insulin resistance and hypercholesterolemia; anti-staphylococcal action
L. lactis subsp. *lactis*	Treatment of antibiotic-associated diarrhoea; adhesion to vaginal epithelial cells; nisin production; modulation of brain activity; antimicrobial activity
L. casei	Treatment of functional constipation in adults; treatment of *C. difficile*-associated diarrhoea; restoration of vaginal flora of patient with bacterial vaginosis; reduction of irritable bowel syndrome symptoms; reduction of diarrhea duration of antibiotic-associated diarrhoea in geriatric patient; immunomodulatory mechanisms; improvement of rheumatoid arthritis status; protection against *Salmonella* infection; prevention of *Salmonella*-induced synovitis; treatment of intravaginal staphylococcosis
Enterococcus durans	Antibiotic and antioxidant activity; adherence to colonic tissue and anti-inflammatory activity
E. faecium	Treatment of antibiotic-associated diarrhea; efficient animal probiotic
Pediococcus acidilactici	Pediocin production with antimicrobial and probiotic properties; bacteriocin production; elimination of *H. pylori* infections

The probiotic LAB play an important role in improvement of intestinal microbiota, enhancement of immune response, cancer prevention, treatment of virus or bacteria infectious in GI tract, and cholesterol-lowering as described below.

Cholesterol-lowering properties

Cholesterol-lowering activity is one of the most promising properties of probiotic LAB via many mechanisms, including (A) increasing fecal bile acid excretion as formed the bile salt deconjugation by LAB bile salt hydrolase BSH) enzyme that leaded to produce bile salts from serum cholesterol and co-precipitated with intestinal cholesterol at pH below 5 before elimination, (B) assimilating cholesterol during the LAB growth, (C) the down-regulation of cholesterol transport (NPC1L1 protein) on small-intestine membrane, and (D) binding of cholesterol to the cellular surface that the cell membranes of resting and dead probiotic cells retain the ability to bind cholesterol thus removed cholesterol from the medium as indirect and direct mechanisms of LAB that exhibited the anti-cholesterolemic effect by *in vitro* and *in vivo* studies (Tsai *et al.*, 2014; Michael *et al.*, 2017; Ma *et al.*, 2019; M. G. Shehata *et al.*, 2019) as described below and shown in Table 2.6.

Table 2.6 Probiotic LAB with hypocholesterolemic effects

Species	Mechanism	References
Lactobacillus acidophilus	BSH	Lin *et al.*, 2014
L. brevis	BSH	da Silva Ferrari *et al.*, 2016
L. casei	BSH, CA	Xiong *et al.*, 2017; Albano *et al.*, 2018
L. delbrueckii subsp.*bulgaricus*	BSH, CA	McAuliffe *et al.*, 2005; Ziarno, 2009
L. fermentum	BSH, CA, Caco-2	Palaniyandi *et al.*, 2019
L. gasseri	BSH	Rani *et al.*, 2017
L. helveticus	BSH, CA, Caco-2	Damodharan *et al.*, 2016
L. paracasei	CA, BSH	Albano *et al.*, 2018; Ghosh *et al.*, 2019
L. pentosus	CA, BSH, Caco-2	Bendali *et al.*, 2017; Choudhary *et al.*, 2019
L. plantarum	CA, BSH, Caco-2	Albano *et al.*, 2018; Yang *et al.*, 2019
L. reuteri	BSH, CA	Martoni *et al.*, 2008; Jones *et al.*, 2012
L. rhamnosus	BSH, CA	M.G. Shehata *et al.*, 2016
Lactococcus lactis subsp. *lactis*	BSH, CA, NPC1L1	M.G. Shehata *et al.*, 2016; M. G. Shehata *et al.*, 2019
Leuconostoc lactis	CA, BSH	Hassanein *et al.*, 2013; Xu *et al.*, 2016
Leuc.mesenteroides	BSH	Xu *et al.*, 2016
Leuc. citreum	BSH	Cho *et al.*, 2015
Pediococcus ethanolidurans	BSH	Xu *et al.*, 2016
P. pentosaceus	BSH	Xu *et al.*, 2016
Enterococcus durans	BSH	Xu *et al.*, 2016
E. faecium	BSH, CA	Xu *et al.*, 2016; Albano *et al.*, 2018
Streptococcus thermophilus	BSH	Tarrah *et al.*, 2018

(A) Bile salt hydrolase (BSH)

BSH (bile salt hydrolase or cholylglycine hydrolase; EC-3. 5. 1. 24) catalyzes the amide bond hydrolysis in conjugated bile salts (CBS) to the free form of deconjugated bile acids (mainly cholic and deoxycholic acid) that released free amino acids (glycine and/ or taurine) as shown in Figure 2.4 and 2.5 (Begley *et al.*, 2006; Ishimwe *et al.*, 2015). BSHs produced by several probiotic bacteria which have the *bsh* gene in the human or animal gastrointestinal tract and nonintestinal LAB that showed the intracellular space and pH 5.0–6.0 as LAB (*Lactobacillus*, *Lactococcus*, etc.) and *Actinobacteria* (*Bifidobacterium* and *Propionibacterium*) (Patel *et al.*, 2010; Rani *et al.*, 2017; Ma *et al.*, 2019; Ru *et al.*, 2019). Many studies reported the distribution of BSH activity in more than 300 LAB, especially the genus *Lactobacillus*.

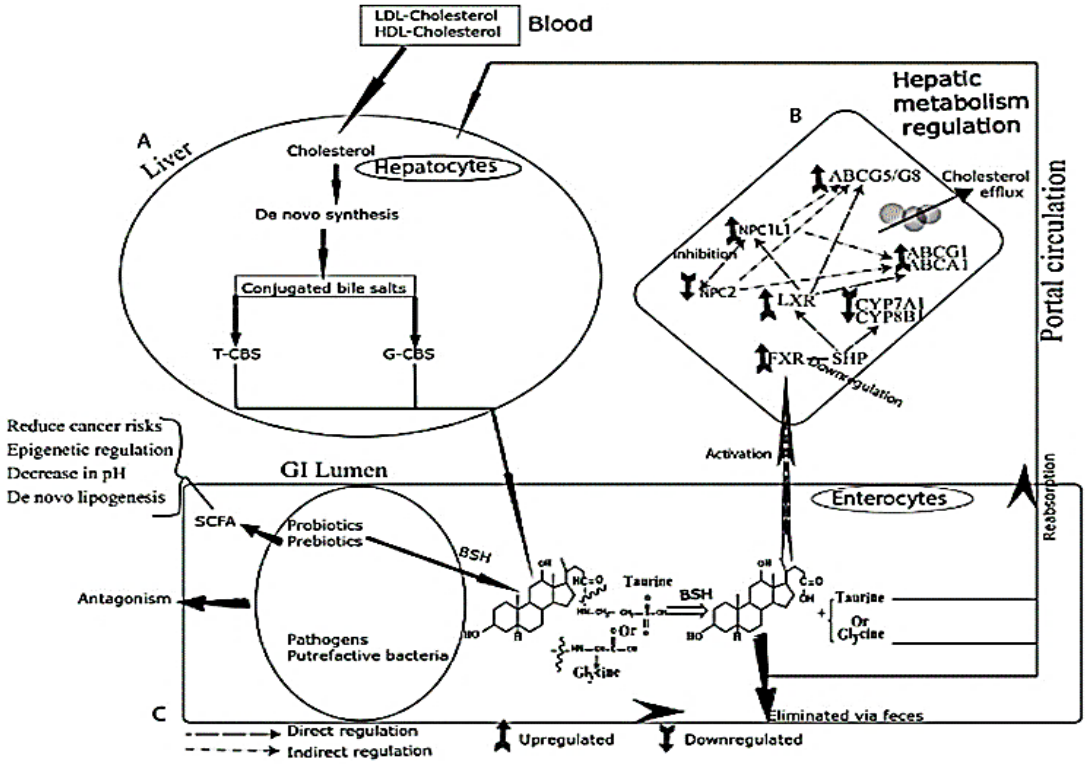

Figure 2.4 Cholesterol circulation and metabolisms with BSH (Ishimwe *et al*., 2015).

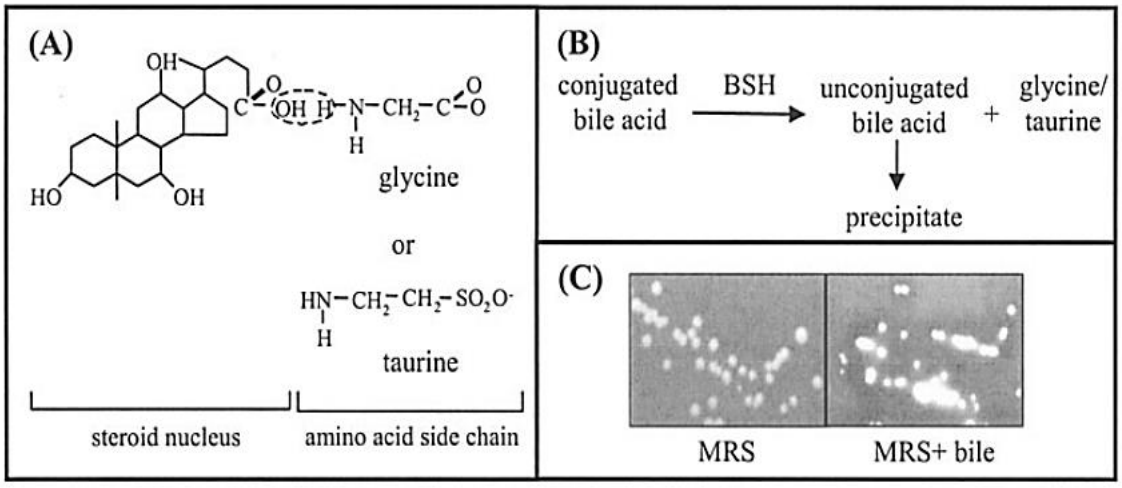

Figure 2.5 Bile salt deconjugation mechanism of bile salt hydrolase (BSH) (Begley *et al.*, 2006)

In the Figure 2.5, (A) show the chemical structure of bile salts that they synthesized in the liver from cholesterol conjugated with either glycine or taurine by an amide bond before secretion to GI lumen. (B) show the catalytic reaction by BSH enzymes that hydrolyzed the peptide linkage of bile salts, which results in removal of the amino acid from the steroid core (free cholesterol). The resulting unconjugated bile acids precipitate the free cholesterol at low pH. (C) show the detection method of BSH activity (Dashkevicz and Feighner, 1989). LAB was grown overnight in MRS broth, was streaked on MRS agar supplemented with 0.2 % (w/v) glycodeoxycholic acid (GDCA) after incubated anaerobically for 48 h. The white precipitates around colonies showed the BSH activity.

(B) Cholesterol assimilation

Cholesterol removal was closely associated with bacterial growth reduced the cholesterol through assimilation in cell membrane by LAB cell growth in the gut. The assimilation of cholesterol could reduce the amount of cholesterol available for absorption by the intestine (Miremadi *et al.*, 2014). The non-growing (resting) and dead cells of probiotic LAB has been assumed that even nonviable cells could be used as cholesterol reducing agents (Tok and Aslim, 2010). It is implied that cholesterol removal is achieved not only through the mechanism of assimilation by growing probiotic cells but also by adhesion to cell membranes. However, the amount of cholesterol removed by live cells was significantly higher than that

removed by dead cells (Kimoto *et al.*, 2007). For the direct method of cholesterol assimilation was determined using scanning electron microscopy examination confirmed the cholesterol-binding activity. The bright zone around the probiotic LAB cells might indicate the secretion of exopolysaccharides that adhere to the cell surface and could absorb cholesterol as a role in cholesterol binding (M. G. Shehata *et al.*, 2019). The indirect method of cholesterol assimilation was determined the remaining cholesterol concentration after incubated LAB in MRS broth supplemented with the water-solubled cholesterol using the modified colorimetric method (Rudel and Morris, 1973; Tomaro-Duchesneau *et al.*, 2014).

Antimicrobial activity

Many studies reported the LAB produced bacteriocins, antibacterial substances with narrow or broad spectrum of activity against spoilage microorganisms and biofilms. Bacteriocin biosynthesis is an important role of disinfect pathogen in fermented foods as well as in the gastrointestinal environment. The antimicrobial activity presented may be association with the production of antimicrobial peptides or bacteriocin-like substances. The antimicrobial effect of LAB was the production of lactic acid that reduced pH as harmful acidic environment. In addition of lactic acid produced various antimicrobial compounds which can be classified to the low molecular mass (LMM) compounds such as hydrogen peroxide, carbondioxide, and bacteriocins (Rabie *et al.*, 2018).

Cytotoxicity

Studies of LAB (*L. acidophilus* and *L. rhamnosus* GG) including their substances exhibited to reduce the risk, incidence and number of cancers that might be involved to decrease levels of several enzymes that leaded to convert pro-carcinogens to carcinogens such as β-glucuronidase, nitroreductase, and azoreductase. Moreover, exopolysaccharides (EPSs) of LAB enhance the immune system that play an important role for regulation of cancer involved with tumor development, apoptosis, and suppression via the cell-mediated immune responses, cytokine pathways, interleukins, and tumor necrosis factors against tumor (Zhong *et al.*, 2014; Tukenmez *et al.*, 2019).

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

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 (CaCO3) (Merck, Germany)
13. Calcium Chloride (CaCl2) (Merck, Germany)
14. Cell line : African green monkey kidney epithelial cells (Vero cells)
15. Cell line : Caco-2 human colon carcinoma cells (ATCC HTB-37)

16. Cell line : Human hepatocellular carcinoma cells (HepG2 ATCCHB-8065)

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 (C6H14) (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)

3.2 Methods

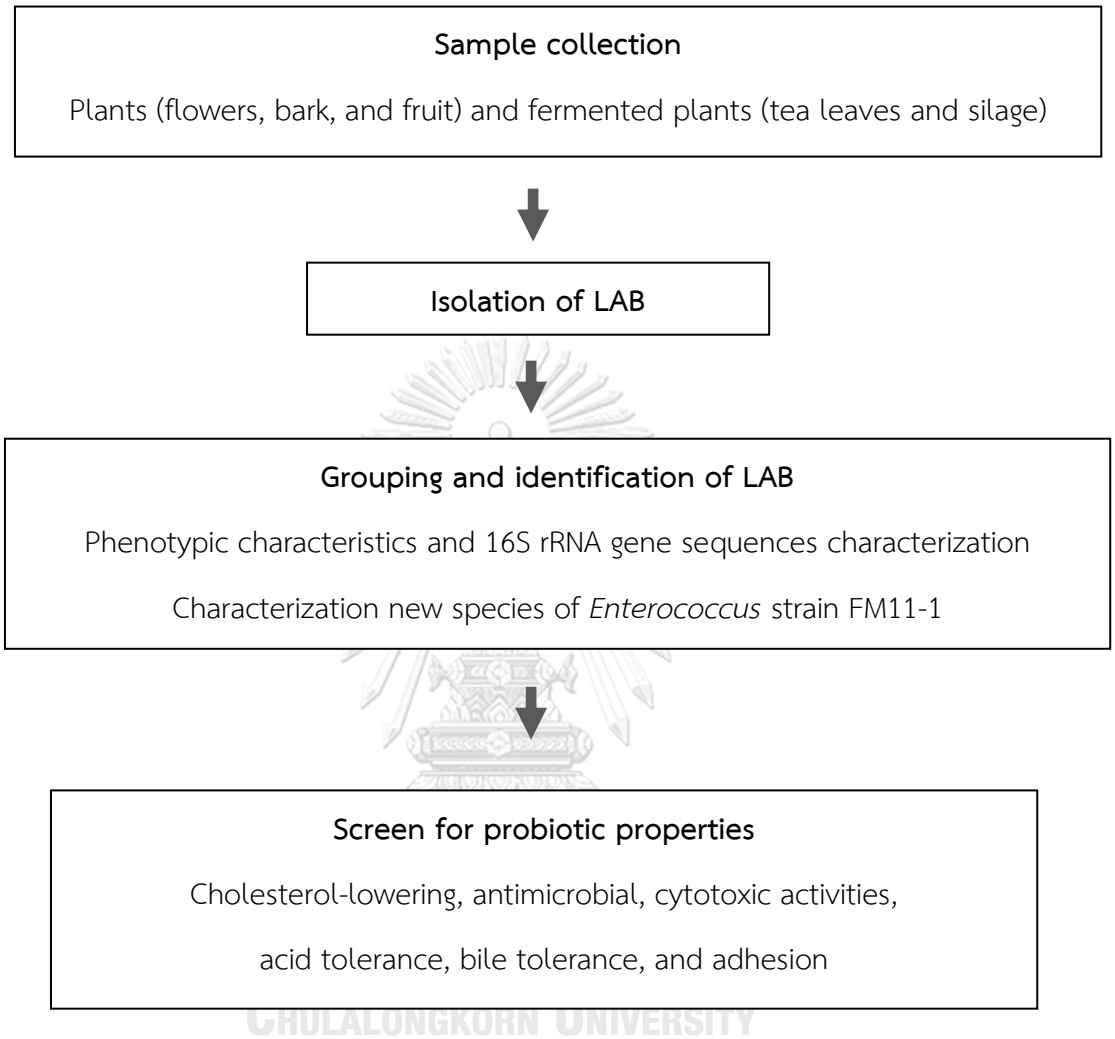

Figure 3.1 The experimental flow chart of this study

3.2.1 Sample collection

LAB were isolated from flowers (9 samples), barks (3 samples), fruit (1 sample) and 12 fermented plant samples fermented tea leaves (10 samples), silage (2 samples). The samples were collected from different provinces, Chiang Rai, Chiang Mai, Yasothon, Nakhon Sawan, Nakhon Nayok, Ratchaburi, Nakhon Si Thammarat district, and Bangkok. (Table 4.1)

3.2.2 LAB isolation

A sample (0.5 g) was enriched in 5 mL de Man Rogosa Sharpe (MRS) broth (Difco, USA) (De Man *et al.*, 1960) and incubated under anaerobic condition using GasPak (Mitsubishi, Japan) at 30 °C for 48-72 h. After the cultivation, one loop of culture broth was streaked on MRS- $CaCO_3$ agar (Appendix A) plate under anaerobic condition at 30 °C for 48 h. The colonies that showed a clear zone were selected and purified on MRS- $CaCO_3$ under anaerobic condition at 30 °C for 48 h. All LAB isolates were kept in 20% glycerol at -80 °C for further experiments. The cultures in 10% skim milk were lyophilized for a long-term preservation.

3.2.3 Grouping and identification of LAB

Preparation of inoculum of isolates

The inoculum of cultures was prepared to be 10^8 CFU/mL by cultivating in MRS broth at 30 °C for 24-48 h under anaerobic condition. Bacterial cells were harvested by centrifugation at 7,000 rpm for 10 min, washed with phosphate saline buffer (1XPBS) three times.

3.2.3.1 Grouping based on phenotypic characteristics

Cell morphology (cell shape, cell arrangement, size, and color) under light microscope and Gram staining was performed. Phenotypic characteristics including catalase test was examined by transferring the colony onto the slide and then 3 % of H_2O_2 was dropped onto the colony (Barrow and Feltham, 2004). Gas production was observed in MRS medium with durham tube. Growth at temperature (15 and 45 °C), pH tolerance (pH 2.0, 4.0, and 9.0), and growth in NaCl concentrations (4.0, 6.0, and 8.0% w/v) were determined as described by Tanasupawat *et al.*, 1998. The hydrolysis of aesculin and arginine were determined as described by Barrow and Feltham, 2004 (Appendix A and B) and nitrate reduction were performed as previous report (Steel *et al.*, 1993) (Appendix B). Acid production from arabinose, cellobiose, fructose, galactose, glucose, lactose, mannose, maltose, mannitol, melibiose, raffinose, rhamnose, ribose, salicin, sorbitol, sucrose, trehalose, and xylose was determined using the basal medium reported by Tanasupawat *et al.*, 2007). The lactic acid isomer was analysed by the enzymatic method (Okada et al., 1978). The

phenotypic characteristics of isolates were used for grouping and the average linkage between groups by the hierarchical cluster was analysed using SPSS 22.0.

Hemolytic activity

The isolates belonged to *Enterococcus* and *Aerococcus* species to determine their hemolytic activity. The selected isolates were streaked on blood agar and incubated at 37 °C for 24 h (Foulquié Moreno *et al.*, 2003). The isolates that produced green-hued zones around the colonies were alpha-hemolysis or those that did not produce any effect on the blood agar were gamma-hemolysis that were considered as non-hemolytic strain. Those producing zones of blood lysis around the colonies were beta-hemolysis as a hemolytic.

3.2.3.2 Identification based on 16S rRNA gene sequence

The represented strains of each group were selected to determine their 16S rRNA gene sequencing. The 16S rRNA gene was amplified by polymerase chain reaction (PCR) technique and were sequenced using the following by the universal primers as primer 20F (5'-AGTTTGATCCTGGCTC-3') and primer 1530R (5'AAGGAGGTGATCCAGCC-3') with amplification reaction (Kawasaki *et al.*, 1993). The sequence of PCR products was carried out by Macrogen, Korea using the universal primers as 27F (5'-AGAGTTTGATCMTGGCTCAG-3'), 518F (5'CCAGCAGCCGCGGTAATACG-3'), 800R (5'-TACCAGGGTATCTAATCC-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3') (Lane, 1991). The alignment has eliminated the gaps and ambiguous nucleotides prior to reconstruct the phylogenetic tree. For multiple alignments of all sequences were performed by the CLUSTAL X version 1.81 in BioEdit software (Thompson *et al.*, 1997). The similarity of 16S rRNA gene sequences was determined using BLAST software compared to the EzBioCloud server database (Yoon *et al.*, 2017). The phylogenetic tree as neighbor-joining (Saitou and Nei, 1987) were reconstructed using MEGA version 7.0 (**S. Kumar *et al.*, 2016**). The confidence values of all branches in the phylogenetic tree were evaluated using the bootstrap resampling method with 1000 replications (Felsenstein, 1985).

3.2.3.3 Characterization of *Enterococcus* strain FM11-1

The candidate novel species of LAB based on the 16S rRNA gene sequence was selected to identify using a polyphasic approach and whole genome sequencing (WGS) compared with the close type strains as showed in the below.

Phenotypic characteristics

Phenotypic characteristics including morphological (cell shape, spore-forming, and cell arrangement), Gram stain reaction and cultural characteristics (color, size, and shape) were determined as previous methods (Tanasupawat *et al.*, 2007). Physiological and biochemical characteristics including acid production by API 50 CH (bioMérieux, France), nitrate reduction, arginine hydrolysis, diacetyl production, pH for growth, temperature for growth, and NaCl tolerance were also determined as reported by (Techo *et al.*, 2016).

Chemotaxonomic characteristics

Chemotaxonomic characteristics including cell wall peptidoglycan *meso*-diaminopimelic acid (*meso*-DAP) and cellular fatty acids were analyzed. The cell wall peptidoglycan was determined using TLC method (Hasegawa *et al.*, 1983; Staneck and Roberts, 1974). The cellular fatty acids were determined by preparing the fatty acid methyl esters (Sasser, 1990) and were analyzed using gas chromatography according to the instructions of the microbial Identification System (MIDI).

Genotypic and phylogenetic analysis

Genomic DNA of the candidate new species LAB was extracted and purified according to the modified methods (Yamada and Komagata, 1970; Tamaoka, 1994). Briefly, cell pellet after incubating in MRS broth at 37°C for 18-24 h was suspended in 5 mL of saline-EDTA with 10 mg of lysozyme and was incubated at 37 °C for 2 h. The mixture was added with 0.1 M Tris and 0.1 M NaCl, pH 9 (2 mL) and 10% sodium dodecyl sulphate (SDS) (0.5 mL) and then heated at 55 °C for 10 min. In order to separate the cell lysates, phenol:chloroform (1:1) solution (5 mL) was added to the mixture, well-mixed, and centrifuged at 10,000 rpm for 10 min. The upper layer of the mixture was collected and transferred to a beaker and then immediately added with 95% of cold ethanol to isolate the DNA. The DNA was spooled with glass rod and placed at room temperature until dry. After that, the DNA was dissolved in 0.3 mL of 0.1x SSC (0.1 M NaCl and 0.015 M Sodium citrate, pH 7). For DNA purification, the sample was added with RNase A solution (0.3 mL) and incubated at 37 °C for 20 min. The DNA sample was added with 10X SSC solution (0.5 mL) and extracted with phenol:chloroform (1:1). The DNA was isolated from the upper layer of the mixture, spooled with a glass rod, dried and re-dissolved in 0.3 mL of 0.1x SSC. The purity of the DNA sample was determined by using spectrophotometer at OD_{260} and OD_{280} nm. The ratio of OD_{260}/OD_{280} should be between 1.8 to 2.0.

The 16S rRNA gene was amplified by the PCR technique. The PCR product was sequenced (Macrogen) using the universal primers: 27F (5'-AGAGTTTGATCMTGGCTCAG-3'), 337F (5'-GACTCCTACGGGAGGCWGCAG-3'), 518F (5'-CCAGCAGCCGCGGTAATACG-3'), 800R (5'-TACCAGGGTATCTAATCC-3), 907R (5'-CCGTCAATTCMTTTRAGTTT-3'), 1100R (5'-GGGTTGCGCTCGTTG-3'), and 1492R (5'-TACGGYTACCTTGTTACGACTT-3). The 16S rRNA gene sequences were aligned with selected sequences obtained from the EzBioCloud server database (Yoon *et al.*, 2017) and NCBI BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) by using by the CLUSTAL X version 1.81 in BioEdit software (Thompson *et al.*, 1997). Phylogenetic trees were constructed based on the neighbour-joining (Saitou and Nei, 1987), maximum-likelihood (Kimura, 1980), and maximum-parsimony (Nei and Kumar, 2000) methods using MEGA 7 software (**S. Kumar *et al.*, 2016**). The confidence values of nodes were evaluated using the bootstrap resampling method with 1000 replications (Felsenstein, 1985). Whole genome sequencing of the candidate new species LAB was performed using the Nextera DNA Flex Library Prep Kit and Illumina MiSeq platform with MiSeq v3 reagent kit (600 cycles). Paired-reads were trimmed and assembled with the CLC Genomics Workbench 11.0.1. Genome annotation was evaluated using the DFAST web service (Tanizawa *et al.*, 2017). Average nucleotide identity (ANI) values of the candidate new species LAB with their closely related type strains were pairwise calculated using ANI-Blast (ANIb) and ANI-MUMmer (ANIm) algorithms (Richter and Rosselló-Móra, 2009) implemented with the JspeciesWS web

service (Richter *et al.*, 2015). The digital DNA-DNA hybridization (dDDH) was calculated with the Genome-to-Genome Distance among the candidate new species LAB and the closely related species using the BLAST+ method (**Meier-Kolthoff *et al.*, 2013b**). The genome length of its incomplete draft genomes was analyzed based on the recommended formula 2 (identities/HSP length).

Genome sequence analysis

The whole genome sequencing of the selected strains was carried out using an Illumina Miseq platform (Illumina) by using 2×250 bp paired end reads. Assembly of the reads to contigs was accomplished by using SPAdes 3.12 (Bankevich *et al.*, 2012). The draft assemblies of them had been submitted to the Gen-Bank and were publicly available. Phylogenomic tree based on whole-genome sequences was constructed on the Type (Strain) Genome Server (TYGS) (Meier-Kolthoff and Goker, 2019). Tree inferred with FastME 2.1.4 (Lefort *et al.*, 2015) from Genome BLAST Distance Phylogeny (GBDP) distances calculated from genome sequences. Branch lengths are scaled in terms of GBDP distance formula d_5. The genomes were annotated using the RAST server followed by the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) and a comparison performed in the SEED Viewer (Aziz *et al.*, 2008; Aziz *et al.*, 2012). Average nucleotide identity (ANI) values were calculated with pairwise genome alignment of the draft genome sequences of related type strains using the ANI-BLAST (ANIb) and ANI-MUMmer (ANIm) algorithms implemented within the JspeciesWS web service (Richter and Rosselló-Móra, 2009; Richter *et al.*,

2015). Calculation of the digital DNA–DNA hybridization (dDDH) values was achieved using the Genome-to-Genome Distance Calculator (GGDC 2.1) using the BLAST+ method (**Meier-Kolthoff *et al.*, 2013a**). *In silico* G+C content of the selected strains was determined according to the genomic DNA sequences. Results were performed using the recommended formula 2 (identities/HSP length), which is useful when dealing with the incomplete draft genomes.

3.2.4 Screening probiotic properties of LAB

3.2.4.1 Bile salt hydrolase for cholesterol-lowering activity

All isolated LAB were determined for bile salt hydrolase (BSH) activity by the qualitative direct plate assay (Ahn *et al.*, 2003; Tsai *et al.*, 2014). Briefly, LAB was inoculated in MRS broth under anaerobic condition at 37 °C for 48 h. After the inoculation, 10 µL of LAB inoculum (10^8 CFU/mL, approximately optical density as 0.7-0.8 at OD 600 nm) were spotted on MRS agar supplemented with 0.5% (w/v) TDCA (Sigma, USA) and 0.37 g/L of $CaCl_2$. All LAB on plates (in triplicate) were incubated under anaerobic conditions at 37 °C for 72 h. The LAB colony showed the precipitation zone of bile acid around the colony (opaque halo) was considered a positive BSH activity.

3.2.4.2 Cholesterol assimilation for cholesterol-lowering activity

All isolated LAB were performed for cholesterol assimilation (CA). Freshly MRS broth supplemented with the 0.22 µm filter sterilized cholesterol PEG-600 (100 µg/mL) were used to inoculate 1% (v/v) of each isolated LAB (10^8 CFU/mL,

approximately optical density as 0.7-0.8 at OD 600 nm) on anaerobic conditions at 37 °C for 48 h. After incubation, all inoculum were centrifuged at 4,000 rpm at 4 °C for 15 min. The supernatant of each isolate was determined the remaining cholesterol concentration using the modified colorimetric method (Rudel and Morris, 1973; Tomaro-Duchesneau *et al.*, 2014). Briefly, 100 µL of each supernatant was transferred to an eppendorf tube and was added with 100 µL of 33% (w/v) KOH and 200 µL of absolute EtOH. The solution was vortexed for 1 min and followed by heating at 60 °C for 15 min. After cooling, 200 µL of deionized water and 500 µL of hexane were added to the solutions and vortexed for 1 min. The mixed solution was stood to separate in two phases at room temperature. Subsequently, 100 µL of the hexane upper layer was transferred to 96-well plates and the solvent was evaporated at room temperature. And then, the absolutely dried sample was added 200 µL of fresh solution of 50 mg/dL O-phthalaldehyde reagent (OPA ; Sigma, USA) in acetic acid and well-mixed under protection from light. After the completely mixing, 50 µL of the concentrated sulphuric acid was added and well-mixed before incubation for 20 min at room temperature under protection from light. The resulting absorbance was read at 550 nm using a Multi-Detection Microplate Reader (USA). The MRS broth supplemented with cholesterol, but no bacteria cells were used as control. The various cholesterol concentration was constructed a standard curve using the cholesterol stock solution. All samples were determined in triplicate and required twice tests. The ability of cholesterol assimilation as by cholesterol removal of each

LAB from media were also calculated in terms of percent cholesterol assimilation by the following formula as in the below. The result of cholesterol assimilation of each sample was exhibited as mean and standard deviation (SD).

3.2.4.3 Antimicrobial activity

LAB was cultivated in MRS broth under anaerobic condition at 37 °C for 48 h. Each inoculum was collected by centrifugation at 10,000 rpm (5810R, Eppendorf) for 5 min. The cell free supernatant broths have adjusted the pH to neutral with 1 N NaOH before the antibacterial study against test microorganisms. The test microorganisms as *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* ATCC 6633, *Kocuria rhizophila* ATCC 9341, *Escherichia coli* ATCC 25922, and *Pseudomonas aeruginosa* ATCC 27853 were cultivated on Tryptic soy agar (TSA) at 37 °C for 24-48 h while *Candida albicans* ATCC 10231 was cultivated on Sabouraud dextrose agar (SDA) at 30 °C for 48 h. After incubation, the suspended test microorganisms (10^8 CFU/mL, standardized the turbidity with McFarland solution No.0.5) in normal saline was used to determine the antibacterial activity of the isolated LAB by agar well diffusion method. The cell suspension was spread on Mueller Hinton agar with a sterilized cotton swab. The plates were allowed to dry, and then a sterile cork borer of diameter (5 mm) was used to cut uniform wells in the agar. Each well was filled with 60 µL of cell free neutral supernatant broths obtained from each LAB. After incubation at 37°C for 48 h, the plates were observed for a zone of inhibition (ZOI) around the well. Results were considered positive if the

diameter (mm) of the ZOI was greater than 1mm. The experiment of antimicrobial activity was carried out in triplicates and was reported as a diameter (mm) of ZOI ± SD. (Cizeikiene *et al.*, 2013)

3.2.4.4 Cytotoxicity

Preparation of LAB

Lactic acid bacteria were selected based on BSH activity, cholesterol assimilation activity, antimicrobial activity, candidate new species, and the representation of LAB groups for cytotoxicity against Caco-2 (colon carcinoma, ATCC HTB-37) and Vero (normal cells from African monkey kidney) cells by MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) based on the capacity for viable cells to metabolize a tetrazolium colorless salt to a blue formazan in mitochondria of viable cells. In brief, the selected LAB was inoculated in MRS broth at 37°C overnight. The inoculum of LAB was diluted in MRS broth (10^8 cells/mL) and was incubated at 37°C for 48 hr. The cell-free supernatant of selected LAB was collected by centrifugation at 7,000 rpm for 10 min and was adjusted the pH to neutral with 1 N NaOH before the cytotoxicity testing. They were concentrated by speed-vacuum drying (Rotational Vacuum Concentrator RVC 2-18, Germany). The residuals were re-suspended in an equal volume of specific culture media.

MTT assay

The supernatant of selected LAB from the above was determined the cytotoxicity against tumor cell lines including Caco-2 cells as compared with Vero cells as previously described (Iyer *et al.*, 2008). Briefly, All cell lines were cultured at 37 °C with humidified atmosphere containing 5% CO_2 in specific culture media supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/mL) and streptomycin (0.1 mg/mL) as Caco-2 cells in Dulbecco modified Eagle's minimal essential medium (DMEM) and Vero cells in Medium 199 (M199). The final density of each cell lines as 1 x 10^5 cells per well were seeded in 96-well plates. Caco-2 and Vero cells were incubated at 37°C overnight before treatment with 10% v/v of LAB supernatant samples. All treated well plates were incubated at 37°C for 24 h in 5% CO_2 incubator before adding the MTT solutions in each well and incubating at 37°C for 3 h in 5% CO_2 incubator. Each well plates have discarded the supernatant before adding dimethyl sulfoxide (DMSO) and mixing gently. The optical density was measured at 570 and 595 nm using a microplate reader. 50 and 25 μM of Cisplatin was used as positive control and MRS broth with cells were used negative control. The experiment was performed in triplicate of three independent assays. The percentage of cell viability was calculated as in the formula below.

$$\text{Percentage of cell viability (\%)} = \frac{\text{Average absorbance of treated cells}}{\text{Average absorbance of negative control}} \times 100$$

3.2.4.5 Acid and bile tolerance

Acid tolerance

All LAB has determined the acid tolerance property according to the method (Hyronimus *et al.*, 2000). Briefly, 100 µL of all each LAB at 10^8 CFU/mL (in duplicates) were cultured in 5 mL of MRS broth which had been adjusted pH with 1M HCl to pH 3.0. The cultures were collected to performed 10-fold serial dilution in phosphate buffer (0.1 M, pH 7.2) before bacterial cell counting at T_0 (the initial count) and were incubated at 37°C for 3 h under anaerobic condition. After incubation, the cultures were performed 10-fold serial dilution in phosphate buffer at T_3 (the contacted count). 10 µL of each serial dilution was transferred onto MRS agar by dropping plate method compared with 0.1 mL of each serial dilution was transferred onto MRS agar by spreading plate method and incubated at 37 °C for 24-48 h under anaerobic condition. Total viable counts determined before incubation (T_0) and after 3 h incubation (T_3) were expressed as the log10 of colonies grown on MRS agar. Unadjusted pH MRS broth (pH 6.5 ± 0.2) was used as a negative control.

Bile tolerance

All LAB has determined the bile tolerance property according to the method (Lee *et al.*, 2011). Briefly, 100 µL of all LAB at 10^8 CFU/mL (in duplicates) were cultured in 5 mL of MRS broth supplemented with bile salt (Oxoid) to achieve 0% bile salt (negative control), 0.3 % bile concentration levels, respectively. The cultures were collected to performed 10-fold serial dilution in phosphate buffer (0.1

M, pH 7.2) before bacterial cell counting at T_0 (the initial count) and were incubated at 37℃ for 3 h under anaerobic condition. After that, 10-fold serial dilution in phosphate buffer (0.1 M, pH 7.2) was performed at T_3 (the contacted count). 10 µL of each serial dilution was transferred onto MRS agar by dropping plate method and were incubated at 37 °C for 24-48 h under anaerobic condition. Total viable counts determined before incubation (T_0) and after 3 h incubation (T_3) were expressed as the log10 of colonies grown on MRS agar.

3.2.4.6 Adhesion assays

The LAB were selected based on their BSH activity, cholesterol assimilation activity, antimicrobial activity, resistance to gastric conditions, and non-hemolytic activity and were inoculated overnight in MRS broth, washed twice with PBS, and re-suspended in DMEM without FBS and antibiotics for the bacterial suspension as concentrations of 10^8 CFU/mL before testing the adhesion assays.

The selected LAB was determined by the adhesion property using Caco-2 intestinal cells using the modified methods (Fernández *et al.*, 2003; Bustos *et al.*, 2012). Briefly, Caco-2 cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and antibiotics at 37 °C in a 5% CO_2 incubator, with changing the medium every two days. The monolayer of Caco-2 cells at a concentration of 5×10^5 cells per well (in duplicate) were seeded in 24-well plate and incubated at 37 °C in a 5% CO_2 incubator. Cells were used at 80 % late post-confluence culture after 15

days. Just before using about 2 h, the monolayer was washed twice with PBS and the culture medium without FBS and antibiotics were added to each well. The bacterial suspensions were added to well plate containing Caco-2 cells and then incubated at 37 °C in a 5% CO_2 incubator for 1 h. The cell cultures were washed twice with PBS and were lysed by 0.05% Triton-X100 solution for 10 min. The number of viable LAB adhesion was determined by serial dilutions and drop plate technique on MRS agar plates. *L. rhamnosus* GG was used as a positive control.

Statistical analysis

Statistical analysis was conducted by one way ANOVA of Tukey method using the SPSS version 22.0. A probability of $P < 0.05$ was considered to be significant.

CHAPTER IV

RESULTS AND DISCUSSIONS

4.1 Isolation of lactic acid bacteria

Seventy lactic acid bacteria were obtained from various sources in Thailand (Table 4.1). They were isolated from flowers (17 isolates), barks (7 isolates), fruit (2 isolates), and Thai fermented plants, including fermented tea leaves " Tea leaves" (31 isolates) and "silage" (13 isolates). They showed the clear zone around the colony on MRS plate supplemented with $CaCO_3$ after incubation at 30 ºC for 48 hr (Figure 4.1). All isolates were preserved in 20 % glycerol at -80 °C and 10 % skim milk as lyophilization.

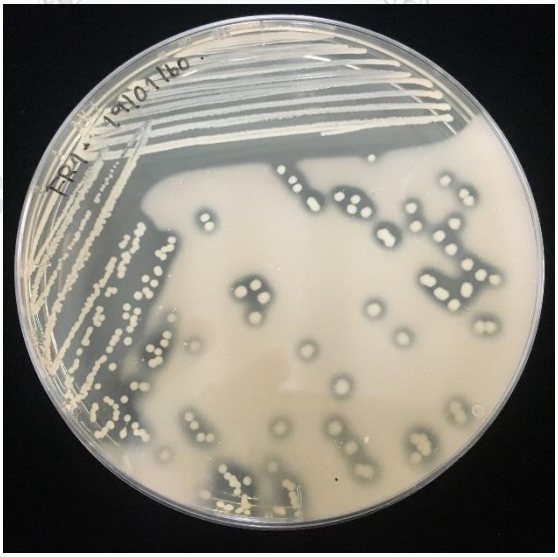

Figure 4.1 The clear zone around the colony of lactic acid bacteria on MRS agar supplemented with $CaCO_3$ after incubation at 30 ºC for 48 hr

Table 4.1 Sample, strain number and number of seventy isolates LAB

Sample	Scientific name	Province	Isolate no.	No. of isolate
Flowers				17
	Gardenia jasminoides	Nakhon Si Thammarat	FM1-1, FM1-2	
	Ixora lucida		FM2-3	
	Wrightia religiosa		FM3-1, FM4-1, FM4-2	
	Ipomoea pes-caprae		FM9-2	
	Solanum torvum		FM11-1, FM11-2, FM11-3	
	Hibiscus syriacus		FM12-1, FM12-2	
	Leucaena leucocephala		FM13-1	
	Jatropha podagrica	Bangkok	FM14-1, FM14-2	
	Tabernaemontana divaricata		FM16-1, FM16-2	
Barks				7
	Oryza sativa	Yasothon	RYM18-2, RYM19-1, RYM20-1, RYM21-1, RYM21-2	
	Terminalia catappa	Chiang Mai	BCM22-2	
	Tamarindus indica		BCM23-3	
Fruit				2
	Diospyros decandra	Ratchaburi	FM15-1, FM15-2	
Fermented plants				44
	Camellia sinensis (Tea leaves)	Chiang Mai	CM24-2, CM24-3, CM24-4, CM25-2, CM25-3, CM26-1, CM27-1, CM28-3, CM33-1, CM38-1, CM38-2, CM38-3	
		Chiang Rai	CRM39-1, CRM39-2, CRM40-3, CRM41-1, CRM41-2, CRM42-1, CRM44-2, CRM45-2, CRM45-3, CRM46-3, CRM47-3, CRM50-3, CRM51-2, CRM54-3, CRM55-1, CRM55-2, CRM56-1, CRM56-2, CRM58-1	
	Pennisetum purpureum (Napier silage)	Nakhon Sawan	NWM59-1, NWM59-2, NWM59-3, NWM60-1, NWM60-2, NWM60-3	
	Zea mays (Corn silage)	Nakhon Nayok	NKM61-1, NKM61-2, NKM62-1, NKM62-2, NKM62-3, NKM63-1, NKM63-2	

4.2 Grouping and identification of isolates

4.2.1 Grouping of isolates

Seventy lactic acid bacteria were isolated from nine flowers (17 strains), three barks (7 strains), one fruit (2 strains), and ten tea leaves (31 strains) and two silage samples (13 strains). They were divided into 11 groups, Group I, II, and III were from flowers; Group IV, V, and VI **from** fruit and barks and Group VII, VIII, IX, X, XI, and XII were from fermented plants based on the phenotypic characteristics, including morphological, physiological, and biochemical characteristics as shown in Table 4.2 Their different characteristics and the hierarchical cluster based on dendrograms using program SPSS 22.0 are showed in Figure 4.2 to 4.5.

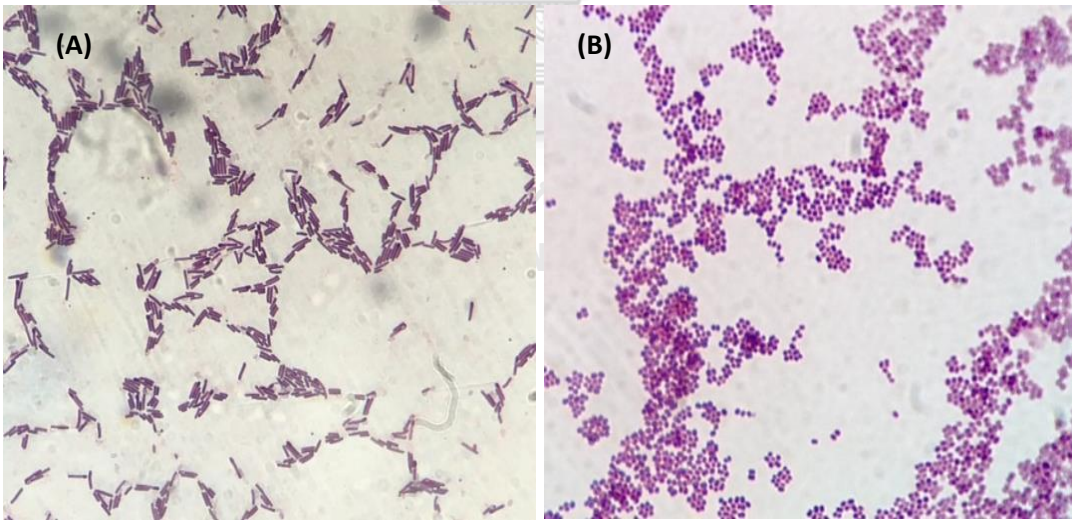

Figure 4.2 Photomicrograph of strain FM3-1 (A) and strain BCM23-3 (B) (x1000).

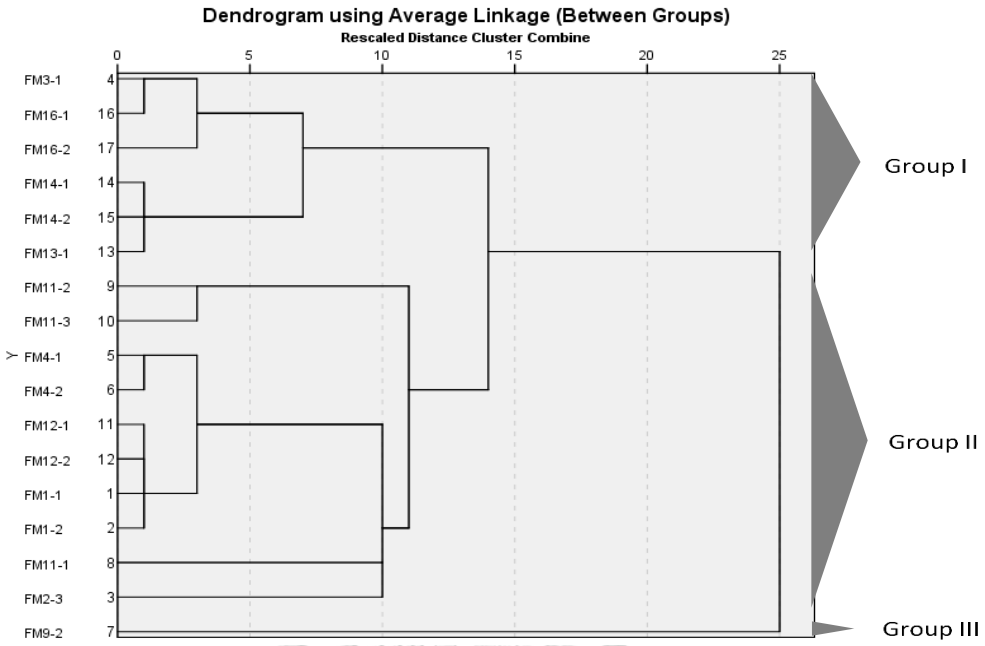

Figure 4.3 The hierarchical cluster based on dendrogram of 17 strains in Group I, II, and III.

Group I consisted of 6 rod-shaped strains (FM3-1, FM13-1, FM14-1, FM14-2, FM16-1, and FM16-2) from flowers. They produced no gas from glucose and did not reduce nitrate. They hydrolyzed aesculin and grew at pH 2.0, 9.0, and in 6 % NaCl. They produced acid from arabinose, cellobiose, fructose, galactose, glucose, lactose, mannose, maltose, mannitol, melibiose, raffinose, rhamnose, ribose, salicin, sorbitol, sucrose, trehalose, and xylose. Variable characteristics are in Table 4.2.

Group II consisted of 10 cocci-shaped strains (FM1-1, FM1-2, FM2-3, FM4-1, FM4-2, FM11-1, FM11-2, FM11-3, FM12-1 and FM12-2) from flower. They produce no gas from glucose and did not reduce arginine. They grew at 45 ºC, pH 2.0, 9.0, and in 6 % NaCl. They produced acid from arabinose, cellobiose, fructose, galactose, glucose, lactose, mannose, maltose, mannitol, melibiose, raffinose, ribose, salicin, sorbitol, sucrose, trehalose, and xylose. Other characteristics are shown variable among them.

Group III consisted of 1 tetrad-shaped strain (FM9-2) from flower sample. They produce no gas from glucose, did not hydrolyzed aesculin and arginine and did not reduce nitrate. They grew at 15, 45 ºC, pH 9.0, and in 6 % NaCl but did not grew at pH2 and in 8% NaCl. They produced acid from arabinose, cellobiose, fructose, galactose, glucose, maltose, ribose, and xylose.

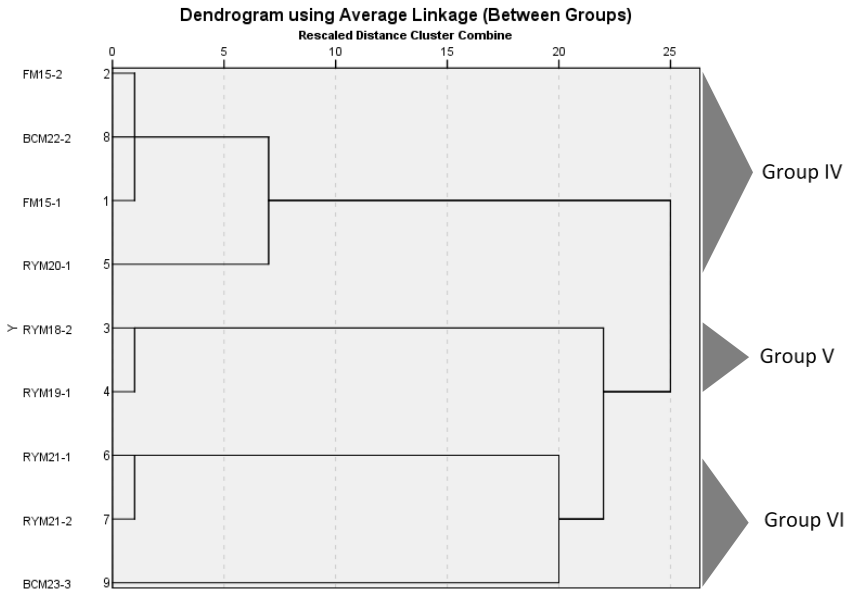

Figure 4.4 The hierarchical cluster based on dendrogram of 10 strains in Group IV, V, and VI.

Group IV consisted of 4 rod-shaped strains (FM15-1, FM15-2, RYM20-1, and BCM22-2) from fruit and bark. They produce no gas from glucose and did not reduce nitrate. They hydrolyze aesculin and arginine and grew at 45 ºC, pH 2.0, 9.0, and in 6 % NaCl. They produced acid from arabinose, cellobiose, fructose, galactose, glucose, lactose, mannose, maltose, mannitol, melibiose, raffinose, rhamnose, ribose, salicin, sorbitol, sucrose, trehalose, and xylose.

Group V consisted of 2 cocci-shaped strains (RYM18-2 and RYM19-1) from bark. They did not produce gas from glucose and did not reduce nitrate. They hydrolyze aesculin and arginine and grew at 45 ºC, and in 6, 8 % NaCl but did not grow at pH 2.0 and 9 ºC. They produced acid from arabinose, cellobiose, fructose, galactose, glucose, lactose, mannose, maltose, mannitol, melibiose, raffinose, rhamnose, ribose, salicin, sorbitol, sucrose, trehalose, and xylose.

Group VI consisted of 3 cocci-shaped strains (RYM21-1, RYM21-2, and RYM23-3) from bark. They did not produce gas from glucose and did not reduce nitrate. They hydrolyze aesculin and arginine and grew at pH 2 and in 6 % NaCl but did not grow at pH 9.0 and in 8 % NaCl. They produced acid from arabinose, cellobiose, fructose, galactose, glucose, lactose, mannose, maltose, mannitol, melibiose, raffinose, rhamnose, ribose, salicin, sorbitol, sucrose, trehalose, and xylose. Other characteristics are shown variable among them.

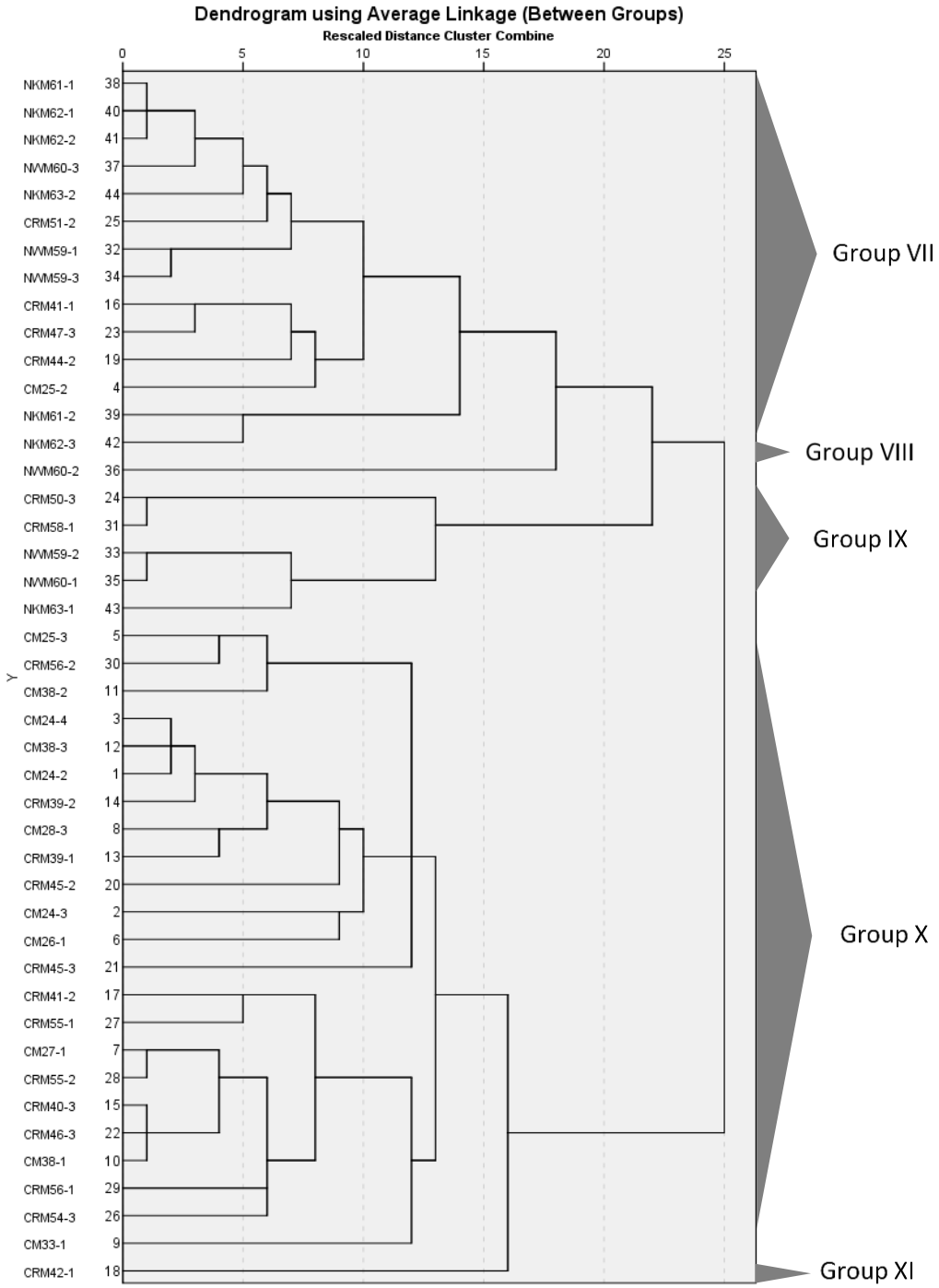

Figure 4.5 The hierarchical cluster based on dendrogram of 44 strains in Group VII, VIII, IX, X, and XI

Group VII consisted of 14 rod-shaped strains (CM25-2, CRM41-1, CRM44-2, CRM47-3, CRM51-2, NWM59-1, NWM59-3, NWM60-3, NKM61-1, NKM61-2, NKM62-1, NKM62-2, NKM62-3, and NKM63-2) from fermented plant as tea leaves and silage. They did not produce gas from glucose. These strains grew at pH 2.0. They produced acid from arabinose, cellobiose, fructose, galactose, glucose, lactose, mannose, maltose, mannitol, melibiose, raffinose, rhamnose, ribose, salicin, sorbitol, sucrose, trehalose, and xylose. Other characteristics are shown variable among them.

Group VIII consisted of 1 rod-shaped strain (NWM60-2) from fermented plant as silage. They produce gas from glucose. It did not used arginine. This hydrolyze aesculin and grew pH 2.0, 9.0, and in 6 % NaCl but did not grew at 45 °C and in 8 % NaCl. This produced acid arabinose, cellobiose, fructose, galactose, glucose, maltose, ribose, xylose and nitrate.

Group IX consisted of 5 rod-shaped strains (CRM50-3, CRM58-1, NWM59-2, NWM60-1, and NKM63-1) from fermented plant as tea leaves and silage. They product no hydrolyze aesculin and did not reduce nitrate. They produce gas from glucose and grew at 45 °C and pH 9.0. They produced acid fructose and ribose. They did not produce acid arabinose, cellobiose, galactose, glucose, lactose, mannose, maltose, mannitol, melibiose, raffinose, rhamnose, salicin, sorbitol, sucrose, trehalose, xylose, and arginine. Other characteristics are shown variable among them.

Group X consisted of 23 rod-shaped strains (CM24-2, CM24-3, CM24-4, CM25-3, CM26-1, CM27-1, CM28-3, CM33-1, CM38-1, CM38-2, CM38-3, CRM39-1, CRM39-2, CRM40-3, CRM41-2, CRM45-2, CRM45-3, CRM46-3, CRM54-3, CRM55-1, CRM55-2, CRM56-1, and CRM56-2) from fermented plant as tea leaves. They produce no gas from glucose and did not used arginine. They hydrolyze aesculin and grew at 45 ºC, pH 2.0, 9.0, and in 6 % NaCl. They produced acid arabinose, cellobiose, fructose, galactose, glucose, lactose, mannose, maltose, mannitol, melibiose, raffinose, rhamnose, ribose, salicin, sorbitol, sucrose, trehalose, and xylose. Other characteristics are shown variable among them.

Group XI consisted of 1 tetrad-shaped strain (CRM42-1) from fermented plant tea leaves. They did not produce gas from glucose, did not used arginine and did not reduce nitrate. They hydrolyze aesculin and grew at 45 ºC, and in 6, 8 % NaCl but did not grew at pH 2.0 and pH 9.0. They could. They produced acid arabinose, cellobiose, fructose, galactose, glucose, lactose, mannose, maltose, mannitol, melibiose, raffinose, rhamnose, ribose, salicin, sorbitol, sucrose, trehalose, and xylose.

Table 4.2 The phenotypic characteristics of 11 groups

Characteristics	Group I	Group II	Group III	Group IV	Group V	Group VI
No. of isolate	**6**	**10**	**1**	**4**	**2**	**3**
Cell Shape	Rods	Cocci	Tetrads	Rods	Cocci	Cocci
Gas from glucose	-	-	-	-	-	-
Arginine	v	-	-	+	+	V
Aesculin	+	-	-	+	+	V
Nitrate	-	v	-	-	-	-
Growth in 6% NaCl	+	+	+	+	+	+
8% NaCl	-	v	-	-	+	-
Growth at pH 2	+	+	-	+	-	+
pH 9	+	+	+	+	-	-
Growth at 45 °C	v	+	+	+	+	+
Acid from:						
Arabinose	+	+	+	+	+	+
Cellobiose	+	+	+	+	+	+
Fructose	+	+	+	+	+	+
Galactose	+	+	+	+	+	+
Glucose	+	+	+	+	+	+
Lactose	+	+	-	+	+	+
Mannose	+	+	-	+	+	+
Maltose	+	+	+	+	+	+
Mannitol	+	+	-	+	+	+
Melibiose	+	+	-	+	+	+
Raffinose	+	+	-	+	+	+
Rhamnose	+	V	-	+	+	+
Ribose	+	+	+	+	+	+
Salicin	+	+	-	+	+	+
Sorbitol	+	-	-	+	+	+
Sucrose	+	+	-	+	+	+
Trehalose	+	+	-	+	+	+
Xylose	+	+	+	+	+	+

+, positive reaction; -, negative reaction.

Table 4.2 The phenotypic characteristics of 11 groups (continued)

Characteristics	Group VII	Group VIII	Group IX	Group X	Group XI
No. of isolate	14	**1**	5	23	1
Shapes	Rod	Rod	Rod	Rod	Tetrads
Gas from glucose	-	-	+	-	-
Arginine	v	-	-	-	-
Aesculin	v	+	v	+	+
Nitrate	v	+	-	V	-
Growth in 6% NaCl	v	+	-	+	+
8% NaCl	v	-	-	V	+
Growth at pH 2	+	+	V	+	-
pH 9	v	+	+	+	-
Growth at 45 °C	v	-	+	+	+
Acid from:					
Arabinose	+	+	v	+	+
Cellobiose	+	+	-	+	+
Fructose	+	+	+	+	+
Galactose	+	+	-	+	+
Glucose	+	+	v	+	+
Lactose	+	+	-	+	+
Mannose	+	+	v	+	+
Maltose	+	+	v	+	+
Mannitol	+	+	-	+	+
Melibiose	+	+	-	+	+
Raffinose	+	+	-	+	+
Rhamnose	+	+	-	+	+
Ribose	+	+	+	+	+
Salicin	+	+	-	+	+
Sorbitol	+	+	-	+	+
Sucrose	+	+	-	+	+
Trehalose	+	+	-	+	+
Xylose	+	+	v	+	+

+, positive reaction; -, negative reaction.

4.2.2 Identification of isolates

Forty-two representative strains of each groups were identified based on 16S rRNA gene sequence. Thirty rod-shaped isolates were belonged to *Lactobacillus* including FM3-1, FM14-1, FM14-2, FM15-2, FM16-1, FM16-2, RYM20-1, BCM22-2, CM24-4, CM25-3, CM26-1, CM27-1, CM28-3, CM33-1, CM38-1,CRM39-1, CRM41-1, CRM41-2, CRM46-3, CRM51-2, CRM56-2, NWM59-3, NWM60-2, NKM63-1, NKM61-1, NKM61-2, NKM62-1, NKM62-2, NKM63-2, and NWM60-1). Nine cocci isolates were *Enterococcus* including FM1-1, FM1-2, FM12-1, FM2-3, FM4-2, FM11-2, FM11-1, RYM21-2, and BCM23-3), 2 isolates were *Pediococcus* (FM9-2 and CRM42-1), and one isolate was *Aerococcus* RYM18-2) as shown in Figure 4.6 and Table 4.3.

Twelve rod-shaped isolates were isolated from flowers (FM14-1, and FM14-2), fermented tea leaves (CM25-3, CM24-4, CM27-1, CM33-1, CRM39-1, CRM41-1, CRM41-2, and CRM51-2), and silages (NWM59-3 and NKM63-1). They produced DL-lactic acid and contained *meso*-DAP in cell wall. They grew at 15-45 ºC, pH 2.0, 9.0 and in 6-8% NaCl. They produced acid from arabinose, cellobiose, fructose, galactose, glucose, lactose, mannose, maltose, mannitol, melibiose, raffinose, rhamnose, ribose, salicin, sorbitol, sucrose, trehalose, and xylose. They were identified as *L. pentosus* (99.86-100 % similarity) (Zanoni *et al.*, 1987). This species were also found in flower (Frediansyah *et al.*, 2019) and fermented tea leaves (Tanasupawat *et al.*, 2007).

Nine rod-shape isolated from flowers (FM3-1, FM16-1, FM16-2, and FM15-2) bark (BCM22-2), fermented tea leaves (CM26-1, CM28-3, and CRM56-2) and silages (NWM60-2). They produced DL-lactic acid isomers. They grew at at 45 ºC pH 2.0, 9.0 and in 6 % NaCl. They produced acid from arabinose, cellobiose, fructose, galactose, glucose, lactose, mannose, maltose, mannitol, melibiose, raffinose, rhamnose, ribose, salicin, sorbitol, sucrose, trehalose, and xylose. Almost all of them did not produce gas from glucose, did not used aesculin, arginine, and did not reduce nitrate. They were identified as *L. plantarum* subsp. *plantarum* (99.37-100% similarity) (Bringel *et al.*, 2005). This species were also found in flowers, fermented plant tea leaves and silage (Tanasupawat *et al.*, 2007; Ruiz Rodriguez *et al.*, 2019; Sifeeldein *et al.*, 2019).

Five rod-shape isolated from silages (NKM61-1, NKM61-2, NKM62-1, NKM62-2, and NKM63-2). They produced DL-lactic acid. They could hydrolyze aesculin and arginine. They grew at 15-45 ºC, pH 2.0, 9.0, and in 6% NaCl. They produced acid from arabinose, cellobiose, fructose, galactose, glucose, lactose, mannose, maltose, mannitol, melibiose, raffinose, rhamnose, ribose, salicin, sorbitol, sucrose, trehalose, and xylose. Almost all of them did not produce gas from glucose and did not reduce nitrate. They were identified as *L. paracasei* subsp. *tolerans* (99.51-99.93% similarity). (M. D. Collins *et al.*, 1989). This species were also found in flower leaves of fermented plate silage (Doi *et al.*, 2013).

The rod-shaped, strain RYM20-1 isolated from bark produced L- lactic acid isomer. It did not contain *meso*-DAP. It hydrolyzed aesculin and arginine. It grew at 15-45 ºC pH 2.0, 9.0 and in 6 % NaCl. This strain produced acid from arabinose, cellobiose, fructose, galactose, glucose, lactose, mannose, maltose, mannitol, melibiose, raffinose, rhamnose, ribose, salicin, sorbitol, sucrose, trehalose, and xylose. It did not produce gas from glucose and did not reduce nitrate. This strain was identified as *L. kunkeei* (99.93% similarity) (Endo *et al.*, 2012). This species were also found in the flower (Neveling *et al.*, 2012)

The strain CM38-1 was rod-shape isolated from fermented tea leaves. It produced L-lactic acid isomers and contained *meso*-DAP in the cell wall. It hydrolyzed aesculin. It grew at 15-45 ºC pH 2.0, 9.0 and in 6-8 % NaCl. This strain produced acid from arabinose, cellobiose, fructose, galactose, glucose, lactose, mannose, maltose, mannitol, melibiose, raffinose, rhamnose, ribose, salicin, sorbitol, sucrose, trehalose, and xylose. It did not produce gas from glucose, did not hydrolyze arginine, and did not reduce nitrate. This strain was identified as *L. brevis* (99.93 % similarity) (Bergey *et al.*, 1934). This specie was also found in the fermented tea leaves (Horie *et al.*, 2019).

The rod-shaped, strain CRM46-3 isolated from fermented tea leaves produced DL-lactic acid isomer and contained *meso*-DAP in the cell wall. It hydrolyzed aesculin. It grew at 15-45 ºC pH 2.0, 9.0 and in 6-8 % NaCl. This strain produced acid from arabinose, cellobiose, fructose, galactose, glucose, lactose, mannose, maltose,

mannitol, melibiose, raffinose, rhamnose, ribose, salicin, sorbitol, sucrose, trehalose, and xylose. It did not produce gas from glucose, did not hydrolyze arginine, and did not reduce nitrate. This strain was identified as *L. silagincola* (100% similarity) and was also found in the silage (Tohno *et al.*, 2017).

The rod-shaped, strain NWM60-1 isolated from fermented tea leaves produced DL- lactic acid isomer. It did not contain *meso*-DAP. It hydrolyzed aesculin and produce gas from glucose. It grew pH 2.0, 9.0, and in 6 % NaCl but did not grew at 45 ºC and in 8 % NaCl. This strain produced acid from arabinose, cellobiose, fructose, galactose, glucose, maltose, ribose, and xylose. They could hydrolyze aesculin and reduce nitrate. They did not used arginine. This strain was identified as *L. formosensis* (99.44% similarity) (C.-H. Chang *et al.*, 2015). This specie was also found in the silage (Mangwe *et al.*, 2016).

The coccus shape, strain RYM18-2 was isolated from bark. It grew at 15-45 ºC and in 6-8 % NaCl but did not grew at pH 2.0 and pH 9.0. It produced L-lactic acid isomer that did not contain *meso*-DAP. It could hydrolyze aesculin and could use arginine. This strain produced acid from arabinose, cellobiose, fructose, galactose, glucose, lactose, mannose, maltose, mannitol, melibiose, raffinose, rhamnose, ribose, salicin, sorbitol, sucrose, trehalose, and xylose. It did not produce gas from glucose and did not reduce nitrate. This was identified as *Aerococcus urinaeequi* (99.85% similarity) (Tohno *et al.*, 2014). This specie was also found in urine of a horse (Felis *et al.*, 2005).

The tetrad shape, strain FM9-2 isolated from flower grew at 15-45 ºC, pH 9, and in 6 % NaCl but did not grew at pH 2.0 and in 8% NaCl. It produced DL-lactic acid isomer that did not contain meso-DAP. This strain produced acid from arabinose, cellobiose, fructose, galactose, glucose, maltose, ribose, and xylose. They did not produce gas from glucose, did not used aesculin and arginine and did not reduce nitrate. This was identified as *Pediococcus acidilactici* (99.71% similarity) (Wieme *et al.*, 2012). This specie was also found in rhizosphere of olive trees (Florou-Paneri *et al.*, 2013).

The tetrad shape, strain CM42-1 isolated from fermented tea leaves produced DL-lactic acid isomer. It hydrolyzed aesculin. It grew at 15-45 ºC pH 2.0, 9.0 and in 6-8 % NaCl. This strain produced acid from arabinose, cellobiose, fructose, galactose, glucose, lactose, mannose, maltose, mannitol, melibiose, raffinose, rhamnose, ribose, salicin, sorbitol, sucrose, trehalose, and xylose. It did not produce gas from glucose, did not hydrolyze arginine, and did not reduce nitrate. This was identified as *P. pentosaceus* (100 % similarity) (Mees, 1934). This specie was also found in fermented tea leaves (Tanasupawat *et al.*, 2007).

Three cocci, FM1-1, FM1-2, and FM12-1 isolated from flowers. They were that produced L-lactic acid isomers. They grew at 15-45 ºC, pH 2.0, 9.0, and in 6-8% NaCl that used acid from arabinose, cellobiose, fructose, galactose, glucose, lactose, mannose, maltose, mannitol, melibiose, raffinose, rhamnose, ribose, salicin, sorbitol, sucrose, trehalose, and xylose. Almost all of them did not produce gas from glucose,

did not hydrolyze arginine and aesculin, and did not reduce nitrate. They were identified as *E. durans* (99.63-99.71% similarity) (M.D. Collins *et al.*, 1984). This species were also found in the cheese (Amaral *et al.*, 2017).

The coccus shape, strain FM2-3 isolated from flower produced L-lactic acid isomer. It grew at pH 2.0, 9.0 and in 6 % NaCl. This strain produced acid from arabinose, cellobiose, fructose, galactose, glucose, lactose, mannose, maltose, mannitol, melibiose, raffinose, ribose, salicin, sorbitol, sucrose, trehalose, and xylose. It did not produce gas from glucose, did not hydrolyze aesculin and arginine, and did not reduce nitrate. This was identified as *E. gallinarum* (99.92 % similarity) (M.D. Collins *et al.*, 1984). This specie was also found in human and animal (Layton *et al.*, 2010)

Two cocci, FM4-2 and FM11-2 isolated from flowers produced L-lactic acid isomers. They grew at 15-45 ºC, pH 2.0, 9.0, and in 6-8% NaCl that used acid from arabinose, cellobiose, fructose, galactose, glucose, lactose, mannose, maltose, mannitol, melibiose, raffinose, rhamnose, ribose, salicin, sucrose, trehalose, and xylose. Almost all of them did not produce gas from glucose, did not hydrolyze arginine and aesculin, and did not reduce nitrate. They were identified as *E. lactis* (99.77-99.78 % similarity) and was also found in the Italian raw milk cheeses (Morandi *et al.*, 2012).

The coccus shape, strain FM11-1 isolated from flower. This strain was that produced L-lactic acid isomers. It hydrolyzes aesculin and arginine and reduce nitrate. It grew at 15-45 ºC, pH 2.0, 9.0 and in 6-8 % NaCl. This strain produced acid from arabinose, cellobiose, fructose, galactose, glucose, lactose, mannose, maltose, mannitol, melibiose, raffinose, rhamnose, ribose, salicin, sorbitol, sucrose, trehalose, and xylose. It did not produce gas from glucose. This was identified as *E. faecium* (98.79 % similarity) (Schleifer and Kilpper-Bälz, 1984). This specie was also found in flower (Valenzuela *et al.*, 2012).

The coccus shape, strain RYM21-2 isolated from bark. This strain was that produced DL-lactic acid isomers that did not contain *meso*-DAP. It hydrolyzes aesculin and reduce nitrate. It grew at 15-45 ºC, pH 2.0 and in 6 % NaCl. This strain produced acid from arabinose, cellobiose, fructose, galactose, glucose, lactose, mannose, maltose, mannitol, melibiose, raffinose, rhamnose, ribose, salicin, sorbitol, sucrose, trehalose, and xylose. It did not produce gas from glucose and did not hydrolyzes arginine. This was identified as *E. gilvus* (98.93 % similarity) and was also found in the human clinical specimens (Tyrrell *et al.*, 2002).

The coccus shape, strain BCM23-3 isolated from bark. This strain was that produced DL-lactic acid isomers that did not contain *meso*-DAP. It grew at 15-45 ºC, pH 2 and in 6 % NaCl. This strain produced acid from arabinose, cellobiose, fructose, galactose, glucose, lactose, mannose, maltose, mannitol, melibiose, raffinose, rhamnose, ribose, salicin, sorbitol, sucrose, trehalose, and xylose. It did not produce

gas from glucose and did not hydrolyzes arginine. This strain was identified as *E. faecalis* (100 % similarity) (Schleifer and Kilpper-Bälz, 1984). This specie was also found in flower (Valenzuela *et al.*, 2012).

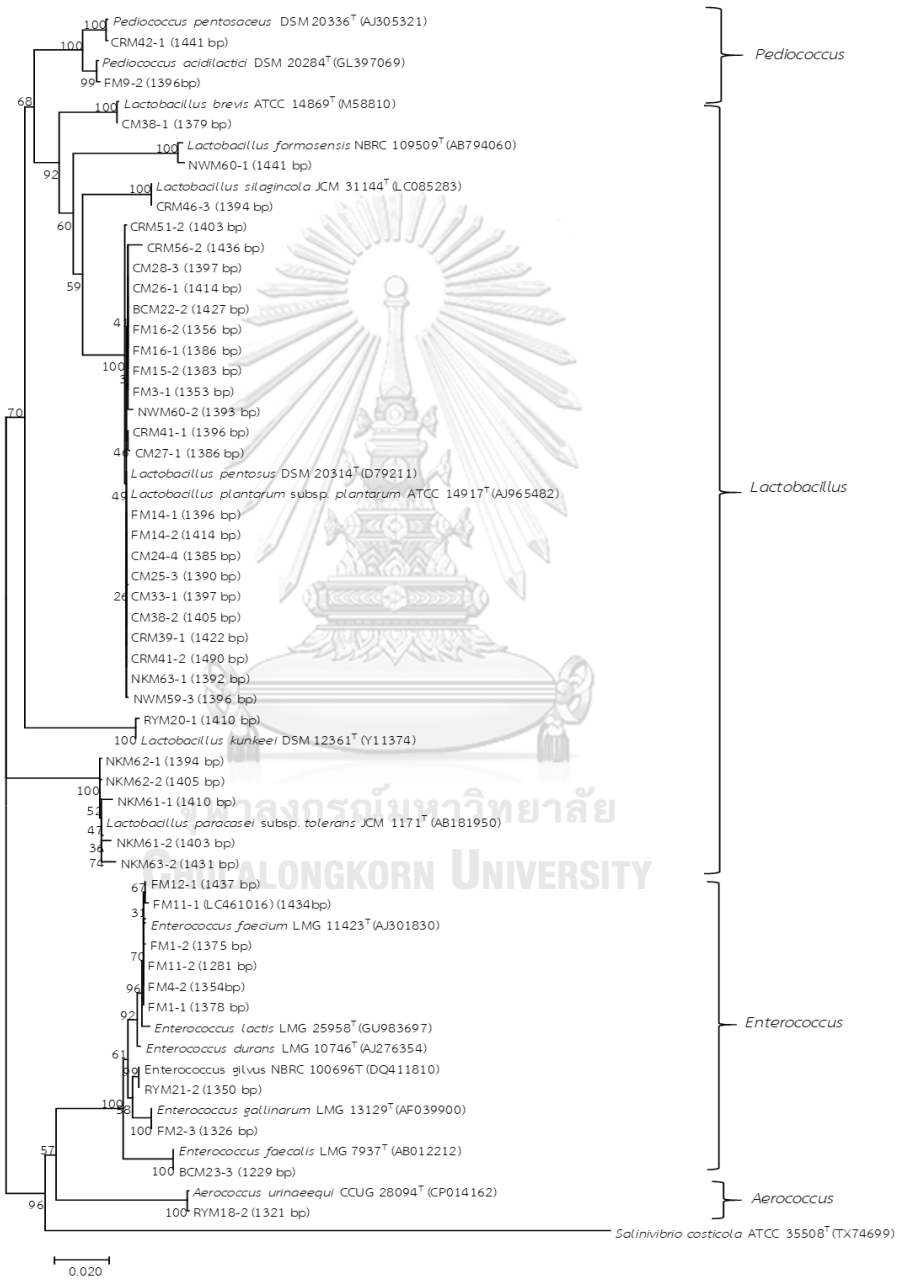

Figure 4.6 Neighbor-joining phylogenetic tree based on 16S rRNA gene sequence of the representative strains and their related type strains.

Table 4.3 16S rRNA gene sequence similarity (%) of the representative strains

Sample	Strain	Closely type strain	Similarity (%)	Length (bp)
Flower				
Gardenia jasminoides	FM1-1	*Enterococcus durans*	99.71	1378
	FM1-2	*Enterococcus durans*	99.63	1375
Ixora lucida	FM2-3	*Enterococcus gallinarum*	99.92	1326
Wrightia religiosa	FM3-1	*Lactobacillus plantarum* subsp. *plantarum*	100	1353
	FM4-2	*Enterococcus lactis*	99.78	1354
Ipomoea pes-caprae	FM9-2	*Pediococcus acidilactici*	99.71	1396
Solanum torvum	**FM11-1**	***Enterococcus faecium***	**98.79**	**1434**
	FM11-2	*Enterococcus lactis*	99.77	1281
Hibiscus syriacus	FM12-1	*Enterococcus durans*	99.65	1437
Jatropha podagrica	FM14-1	*Lactobacillus pentosus*	99.93	1396
	FM14-2	*Lactobacillus pentosus*	100	1414
Tabernaemontana divaricata	FM16-1	*Lactobacillus plantarum* subsp. *plantarum*	100	1383
	FM16-2	*Lactobacillus plantarum* subsp. *plantarum*	99.93	1386
Fruit				
Diospyros decandra	FM15-2	*Lactobacillus plantarum* subsp. *plantarum*	100	1356
Bark				
Oryza sativa	RYM18-2	*Aerococcus urinaeequi*	99.85	1321
	RYM20-1	*Lactobacillus kunkeei*	99.93	1410
	RYM21-2	*Enterococcus gilvus*	99.93	1350
Terminalia catappa	BCM22-2	*Lactobacillus plantarum* subsp. *plantarum*	100	1427
Tamarindus indica	BCM23-3	*Enterococcus faecalis*	100	1229
Fermented plant				
Camellia sinensis	CM24-4	*Lactobacillus pentosus*	100	1385
(Tea leaves)	CM25-3	*Lactobacillus pentosus*	100	1390
	CM26-1	*Lactobacillus plantarum* subsp. *plantarum*	100	1414
	CM27-1	*Lactobacillus pentosus*	99.86	1386
	CM28-3	*Lactobacillus plantarum* subsp. *plantarum*	100	1397
	CM33-1	*Lactobacillus pentosus*	100	1397
	CM38-1	*Lactobacillus brevis*	99.93	1379
	CRM39-1	*Lactobacillus pentosus*	100	1422
	CRM41-1	*Lactobacillus pentosus*	99.93	1396
	CRM41-2	*Lactobacillus pentosus*	100	1490
	CRM42-1	*Pediococcus pentosaceus*	100	1441
	CRM46-3	*Lactobacillus silagincola*	100	1394
	CRM51-2	*Lactobacillus pentosus*	99.93	1403
	CRM56-2	*Lactobacillus plantarum* subsp. *plantarum*	99.37	1436
Pennisetum purpureum	NWM59-3	*Lactobacillus pentosus*	99.93	1396
(Napier silage)	NWM60-1	*Lactobacillus formosensis*	99.44	1393
	NWM60-2	*Lactobacillus plantarum* subsp. *plantarum*	99.57	1441
Zea mays (Corn silage)	NKM61-1	*Lactobacillus paracasei* subsp. *tolerans*	99.64	1410
	NKM61-2	*Lactobacillus paracasei* subsp. *tolerans*	99.71	1403
	NKM62-1	*Lactobacillus paracasei* subsp. *tolerans*	99.93	1394
	NKM62-2	*Lactobacillus paracasei* subsp. *tolerans*	99.71	1405
	NKM63-1	*Lactobacillus pentosus*	100	1392
	NKM63-2	*Lactobacillus paracasei* subsp. *tolerans*	99.51	1431

Table 4.4 Phenotypic characteristics of isolates

Characteristics	*Lactobacillus*							*Aerococcus*
Species	1	2	3	4	5	6	7	8
Cell shape	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Cocci
Gas from glucose	-	-	-	-	-	-	+	-
Growth in 6% NaCl	+	+	+	-	+	+	-	+
Growth in 8% NaCl	+	-	-	-	+	-	-	+
Growth at pH 2	+	+	+	+	+	+	-	-
pH 9	+	+	+	+	+	+	+	-
Growth at 15 °C	+	+	+	+	+	+	+	+
45 °C	+	+	-	+	+	+	+	+
Acid from:								
Arabinose	+	+	+	+	+	+	+	+
Cellobiose	+	+	+	+	+	+	-	+
Fructose	+	+	+	+	+	+	+	+
Galactose	+	+	+	+	+	+	-	+
Glucose	+	+	+	+	+	+	-	+
Lactose	+	+	+	+	+	+	-	+
Mannose	+	+	+	+	+	+	-	+
Maltose	+	+	+	+	+	+	-	+
Mannitol	+	+	+	+	+	+	-	+
Melibiose	+	+	+	+	+	+	-	+
Raffinose	+	+	+	+	+	+	-	+
Rhamnose	+	+	+	+	+	+	-	+
Ribose	+	+	+	+	+	+	+	+
Salicin	+	+	+	+	+	+	-	+
Sorbitol	+	+	+	+	+	+	-	+
Sucrose	+	+	+	+	+	+	-	+
Trehalose	+	+	+	+	+	+	-	+
Xylose	+	+	+	+	+	+	-	+
Aesculin	+	+	-	+	+	+	+	+
Nitrate	-	-	-	-	-	-	-	-
Arginine	-	+	-	+	-	-	-	+
Isomer of lactic acid	DL	DL	DL	L	L	DL	L	L

1; *L. pentosus*; 2; *L. paracasei* subsp. *tolerans*, 3; *L. plantarum* subsp. *plantarum*, 4; *L. kunkeei*, 5; *L. brevis*, 6; *L. silagincola*, 7; *L. formosensis*, 8; *A. urinaeequi*, +; positive, –; negative, R; rods, C; cocci.

Table 4.4 Phenotypic characteristics of isolates (continued)

Characteristics	*Pediococcus*		*Enterococcus*					
Species	9	10	11	12	13	14	15	16
Cell shape	Tetrads	Tetrads	cocci	cocci	cocci	cocci	cocci	cocci
Gas from glucose	-	-	-	-	-	-	-	-
Growth in 6% NaCl	+	+	+	+	+	+	+	+
Growth in 8% NaCl	-	+	+	-	-	+	-	-
Growth at pH 2	-	-	+	+	+	+	+	+
Growth at pH 9	+	-	+	+	+	+	-	-
Growth at 15 °C	+	+	+	-	+	+	+	+
at 45 °C	+	+	+	-	+	+	+	+
Acid from:								
Arabinose	+	+	+	+	+	+	+	+
Cellobiose	+	+	+	+	+	+	+	+
Fructose	+	+	+	+	+	+	+	+
Galactose	+	+	+	+	+	+	+	+
Glucose	+	+	+	+	+	+	+	+
Lactose	-	+	+	+	+	+	+	+
Mannose	-	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	+	+
Mannitol	-	+	+	+	+	+	+	+
Melibiose	-	+	+	+	+	+	+	+
Raffinose	-	+	+	+	+	+	+	+
Rhamnose	-	+	+	-	+	+	+	+
Ribose	+	+	+	+	+	+	+	+
Salicin	-	+	+	+	+	+	+	+
Sorbital	-	+	+	-	-	-	+	+
Sucrose	-	+	+	+	+	+	+	+
Trehalose	-	+	+	+	+	+	+	+
Xylose	+	+	+	+	+	+	+	+
Aesculin	-	+	-	-	-	+	+	-
Nitrate	-	-	-	-	-	+	-	-
Arginine	-	-	-	-	-	+	+	-
Isomer of lactic acid	DL	DL	L	L	L	L	DL	L

9; *P. acidilactici*, 10; *P. pentosaceus*, 11; *E. durans*; 12; *E. gallinarum*, 13; *E. lacti,* 14; *E. faecium*, 15; *E. gilvus*, 16; *E. faecalis*, +; positive, –; negative, T; Tetrads, C; cocci.

4.2.3 Hemolytic type of *Aerococcus* and *Enterococcus* strains

In this study, all isolated LAB in the genus *Aerococcus* (RYM18-2) and *Enterococcus* (9 strains) exhibited that four strains (FM11-2, RYM18-2, RYM21-2, and BCM23-3) showed beta-hemolytic activity as the harmful strains and six strains (FM1-1, FM1-2, FM2-3, FM4-2, FM11-1, and FM12-1) showed alpha-hemolytic activity.

Aerococcus urinaeequi strain RYM18-2 and *Enterococcus lactis* strain FM11-2, *E. gilvus* strain RYM21-2, and *E. faecalis* strain BCM23-3 exhibited completely clear zone on blood agar plate as beta-hemolytic activity.

Enterococcus lactis strain FM4-2, *E. gallinarum* strain FM2-3, *Enterococcus* sp. strain FM11-1, and *E. durans* strain FM1-1, FM1-2, and FM12-1 exhibited green color colony on blood agar plate as alpha-hemolytic activity.

Although *Enterococci* was assessed the type of hemolytic activity on blood agar plates, they are generally nonhemolytic (gamma-hemolytic or no hemolytic strains such as *E. faecalis* and *E. faecium*) as the safety strains and the normal gastrointestinal flora with the resistance to acid and bile salts. In this study, six *Enterococci* were alpha-hemolytic strains while three *Enterococci* were beta-hemolytic strains as the harmful strains. However, reported that used *E. faecium* DSM 7134 at 5×10^8 CFU / kg for feed supplementation with probiotic increase weight performance of primiparous sows. (Franz *et al.*, 2011).

4.3 Characterization of *Enterococcus* strain FM11-1

Strain FM11-1, isolated from the flower of *Solanum torvum* (Family Solanaceae) that collected from Nakhon Si Thammarat province, in the Southern part of Thailand, are Gram-stain-positive, cocci-shape, non-spore-forming, non-motile, and facultative anaerobic bacteria with spherical or ovoid and arranged in pairs or in chains (0.8-1 μm in diameter) as shown in Figure 4.7. Colonies were circular, low-convex, white with entire margins and non-pigmented.

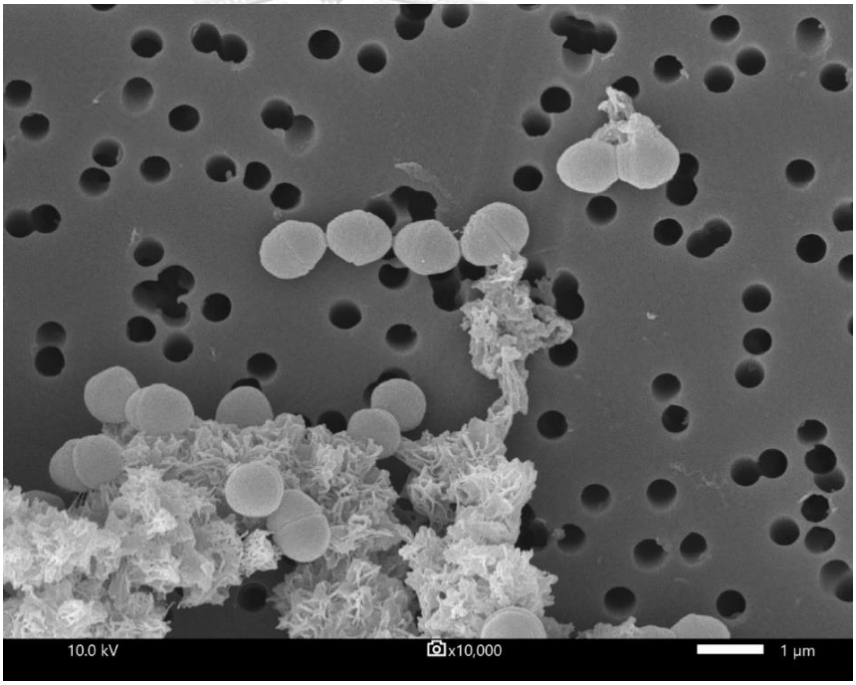

Figure 4.7 Scanning electron micrograph of strain FM11-1 grown in MRS broth at 37 °C for 48 h. Bar, 1 μm.

Catalase activity of strain FM11-1 was negative. This strain did not produce gas from glucose and fermented glucose homofermentative producing only L-isomer of lactic acid. It could not produce diacetyl. It grew well at 10-42 °C with the optimum temperature at 30-37 °C and could grow in the range of pH 6.0-10.0 with optimum growth at pH 6.5-8.0. Growth was observed when strain FM11-1 was cultivated in medium supplemented with 1-7 % (w/v) of NaCl. The different phenotypic characteristics between strain FM11-1 and related strains were described in Table 4.5. The major cellular fatty acid of strain FM11-1 was $C_{19:0}$ cyclo $\omega 8c$ (37.86 %) as shown in Table 4.6. The remainder of fatty acid profile comprised $C_{16:0}$ (15.41 %), Summed Feature 3 ($C_{16:1}$ $\omega 7c$ and/or $C_{16:1}\omega 6c$) (14.93 %), summed feature 8 ($C_{18:1}$ $\omega 7c$ or $C_{18:1}$ $\omega 6c$) (8.93 %), $C_{18:0}$ $\omega 9c$ (5.11 %), $C_{20:0}$ (3.45 %), $C_{18:0}$ (2.15 %), *iso*-$C_{19:0}$ (1.16 %), and $C_{14:0}$ (6.55 %).

The 16S rRNA gene sequence of strain FM11-1 (1434 bp) indicated that the strain belonged to genus *Enterococcus* and was closely related to *E. faecium* NRIC 1145^T (98.79 %), *E. durans* NBRC 100479^T (98.72 %), *E. lactis* LMG 25958^T (98.49 %), and *E. ratti* DSM 15687^T (98.02 %). The phylogenetic tree reconstruction based on 16S rRNA gene sequences showed that strain FM11-1 shared the cluster with *E. faecium* NRIC 1145^T, *E. lactis* LMG 25958^T, and *E. ratti* DSM 15687^T, as the closed relatives, formed a separate branch within the clade of the genus *Enterococcus* (Figure 4.8).

Table 4.5 Differential characteristics of FM11-1 and related type strains.

Strains: 1, Strain FM11-1; 2, *E. faecium* NRIC 1145^T; 3, *E.durans* NBRC 100479^T ; 4, *E.lactis* LMG 25958^T; 5, *E.ratti* DSM 15687^T and +, positive; w, weakly positive; –, negative reaction.

Characteristic	1	2	3	4	5
Temperature range for growth (°C)	10-42	10-37	10-42	10-42	10-42
pH range for growth	6-10	5.5-10	5.5-10	5.5-10	5.5-10
(%) NaCl range for growth	1-6	1-8	1-8	1-8	1-8
API ZYM					
Alkaline phosphatase	+	-	-	-	-
Lipase	-	+	+	-	-
β-Galactosidase	-	+	-	-	-
Acid production from					
Glycerol	+	+	-	-	-
Mannitol	+	+	+	+	w
Mteil-αD-Mannopyranoside	+	-	+	+	+
Mteil-αD-Glucopyranoside	-	-	-	-	w
N-Acetylglucosamine	+	+	-	w	+
Amygdaline	+	-	-	w	w
Arbutin	+	+	w	w	w
Salicin	+	w	-	w	w
D-Melibiose	+	-	-	-	-
D-Trehalose	+	+	+	w	+
Potassium gluconate	-	-	-	-	w

Table 4.6 Cellular fatty acids of FM11-1 and related type strains

Strains: 1, FM11-1; 2, *E. faecium* NRIC 1145^{T}; 3, *E. durans* NBRC 100479^{T} 4, *E. lactis* LMG 25958^{T}; 5, *E. ratti* DSM 15687^{T}. All data are shown as a percentage of the total fatty acids. -, Not detected.

Fatty acid	1	2	3	4	5
Saturated fatty acids					
$C_{9:0}$	-	-	-	-	2.5
$C_{14:0}$	6.6	5.7	7.8	5.9	4.2
$C_{15:0}$	-	-	1.3	-	-
$C_{16:0}$	15.4	33.3	43.8	16.9	36.7
$C_{18:0}$	2.2	1.8	2.8	2.0	3.1
$C_{20:0}$	3.5	-	1.1	1.9	-
Unsaturated fatty acids					
$C_{17:0}$ 2OH	-	1.4	3.0	-	3.2
$C_{18:1}$ *ω9c*	5.1	9.0	11.3	3.8	13.7
$C_{19:0}$ cyclo *ω8c*	37.9	-	-	42.6	-
$C_{20:4}$ *ω6,*9,12,15c	-	-	-	-	1.6
Branched fatty acids					
iso-$C_{16:0}$	-	-	1.6	-	-
iso-$C_{19:0}$	1.2	1.4	3.3	1.3	1.3
Summed Feature 3[a]	14.9	6.9	14.7	12.4	1.1
Summed Feature 7[b]	-	38.1	-	-	32.1
Summed Feature 8[c]	8.9	-	9.3	9.9	-

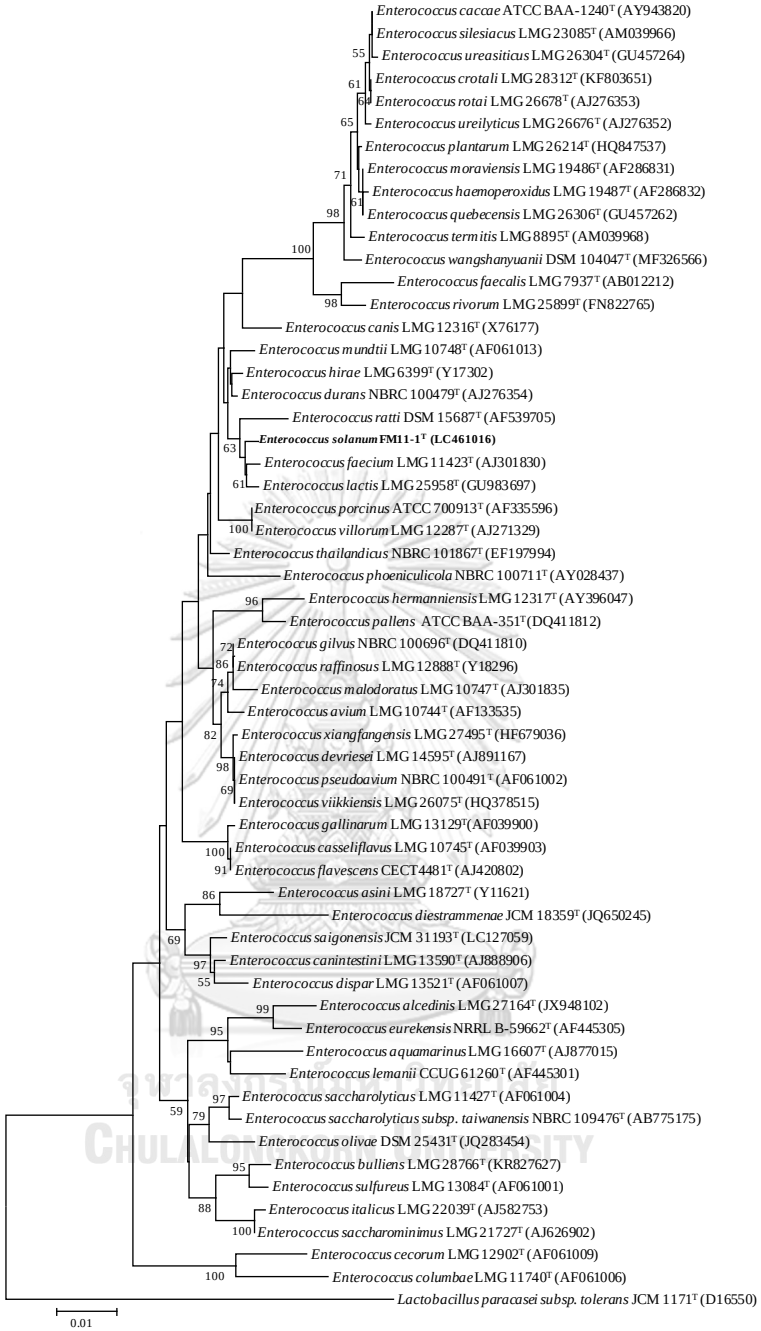

Figure 4.8 Neighbour-joining tree based on 16S rRNA gene sequences of FM11-1 and related species of the genus *Enterococcus*.

Draft genome sequence of strains FM11-1 (BJCC00000000) has 2,784,928 bp in size which was contained 2,586 coding sequences with *in silico* G+C content of 38.1 mol % as in the range of the genus *Enterococcus*. Phylogenomic tree based on TYGS result showing the relationship between the strain FM11-1 with related type strains (Figure 4.9) indicated the strain FM11-1 shared the same node with *E. faecium* NRIC 1145^{T} as provided on 2019-11-08. The ANIb and ANIm values of the draft genomes between strain FM11-1 and the closest type strain as *E. faecium* NRIC 1145^{T} were 93.93 % and 94.87 % that are apparently lower than 95-96 % for the species delineation as shown in Table 4.7. The digital DNA-DNA hybridization (dDDH) between the genomes of the strain FM11-1 and the closest type strain as *E. faecium* NRIC 1145^{T} was 58.3 % (Table 4.7) which was lower than the cut-off value of 70% for species delineation, supporting that strain FM11-1 represents a distinctive novel species in the genus *Enterococcus*.

According to polyphasic characterization and genome analysis, this strain represents a novel species of the genus *Enterococcus*, for which the name *Enterococcus solani* sp. nov. is proposed. The type strain is FM11-1^{T} (=JCM 33322^{T} = LMG 31283^{T}= TISTR 2660^{T}).

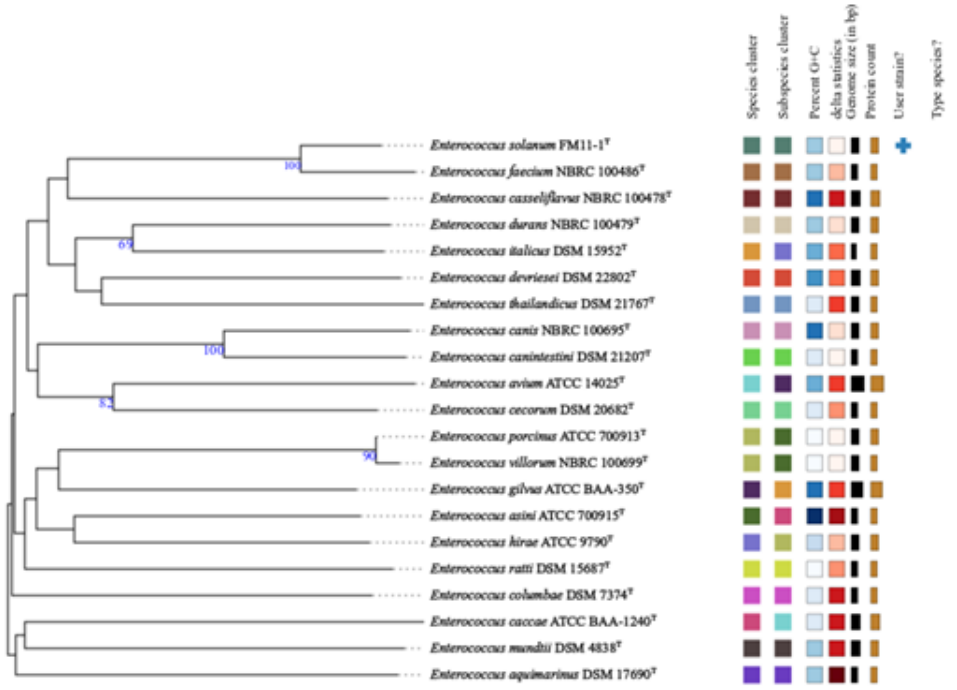

Figure 4.9 Phylogenomic tree based on TYGS result showing the relationship between the strain FM11-1 with related type strains.

Table 4.7 ANIb and ANIm values (%) and the digital (*in silico*) DNA-DNA hybridization (dDDH) values between the draft genomes of strain FM11-1 and its closest related type strains.

Draft genomes: 1, FM11-1^{T} (BKZS00000000); 2, *E. faecium* NRIC 1145^{T} (UFYJ01000001); 3, *E. durans* NBRC 100479^{T} (BCQB00000000), and 4, *E. ratti* DSM 15687^{T} (JXLB01000000)

Query genome	Reference genome	ANIb	ANIm	% dDDH (Formula 2*)	Model C.I.	Distance	Prob. DDH >= 70 %	G+C difference
1	2	93.93	94.87	58.3	55.5 – 61 %	0.0547	46.47	0.03
1	3	77.94	86.70	24.6	22.3 – 27.1 %	0.177	0.01	0.28
1	4	75.98	84.57	21.4	19.2 – 23.9 %	0.2047	0	3.78

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

4.4 Screening probiotic properties of LAB

4.4.1 Bile salt hydrolase for cholesterol-lowering activity

Bile salt hydrolase activity exhibited the precipitation zone around the colony of FM1-1 and FM1-2 on MRS agar supplemented with 0.5 % sodium salt of taurodeoxycholic acid (TDCA) as shown in Figure 4.10.

Twenty-eight strains of all LAB strains, excepted strain RYM 18-2 (as *Aerococcus urinaeequi*), showed the bile salt hydrolase activity on MRS agar supplemented with 0.5 % sodium salt of taurodeoxycholic acid (TDCA), including *Enterococcus durans* strain FM1-1, and FM1-2, *E. gallinarum* strain FM2-3 as strong BSH activity ; *E. lactis* strain FM4-2 and FM11-2 as moderate BSH activity ; *Lactobacillus plantarum* subsp. *plantarum* strain FM3-1, *Pediococcus acidilactici* strain FM9-2, *E. faecium* strain FM11-1, *E. durans* strain FM12-1, *L. plantarum* subsp. *plantarum* strain FM15-2, FM16-2, CM26-1, CM28-3, CRM39-1, and NWM60-2, *L. pentosus* strain FM14-1, CM24-4, CM25-3, CM27-1, CM33-1, and CRM41-1, *Lactobacillus* sp. FM15-1, CM38-3, CRM55-2, and NWM60-3, *L. brevis* strain CM38-1, *Pediococcus pentosaceus* strain CRM42-1, and *L. silaginacola* strain CRM46-3 as weak BSH activity (Table 4.8). These LAB strains were selected to assess the probiotic properties as acid tolerance, bile tolerance, and adhesion assays.

Many *Lactobacillus* strains showed BSH activity such *as L. paracasei* and *L. pentosus*, and *L. plantarum* isolated from dairy products, *L. brevis* isolated from goat dairies showed BSH activity (Table 2.6) while *L. brevis* strain CM38-1 isolated from

fermented tea leaves showed BSH activity and cholesterol assimilation for cholesterol-lowering. In this study. *P. pentosaceus* isolated from fermented tea leaves and *P. acidilactci* isolated from flowers showed BSH activity while other studies as *P. ethanolidurans* and *P. pentosaceus* isolated from stinky soybean and kimchi (Xu *et al.,* 2016).

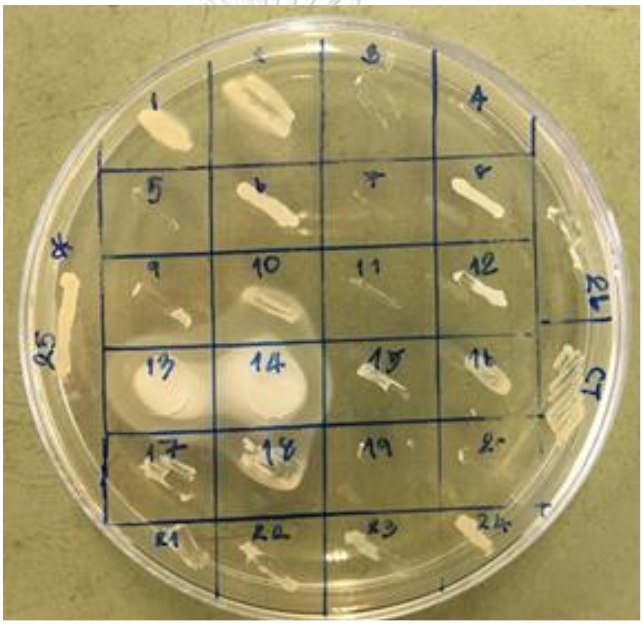

Figure 4.10 Bile salt hydrolase activity showed the precipitation zone around the colony of strains FM1-1 (13) and FM1-2 (14) on MRS agar supplemented with 0.5 % sodium salt of taurodeoxycholic acid (TDCA).

Table 4.8 BSH activity of twenty-eight isolates using agar plate assay

Strain no.	BSH activity
FM1-1, FM1-2, and FM2-3	+++
FM4-2 and FM11-2	++
FM3-1, FM9-2, FM11-1, FM12-1, FM14-1, FM15-1, FM15-2, FM16-2, CM24-4, CM25-3, CM26-1, CM27-1, CM28-3, CM33-1, CM38-1, CM38-3, CRM39-1, CRM41-1, CRM42-1, CRM46-3, CRM55-2, NWM60-2, and NWM60-3	+

+++, strong activity; ++ moderate activity; +, weak activity

4.4.2 Cholesterol assimilation for cholesterol-lowering activity

Nineteen strains showed the percentage of cholesterol assimilation as higher than 45 % on MRS broth supplemented with 100 ug/ml of cholesterol after 48 h incubation.

Enterococcus sp. strain FM11-1 showed the cholesterol assimilation of 46.59 % while *E. lactis* strain FM11-2 showed the cholesterol assimilation of 51.69 %. *Lactobacillus plantarum* subsp. *plantarum* strain FM3-1 showed the cholesterol assimilation of 49.14 %. *L. plantarum* subsp. *plantarum* strains CM28-3, NWM60-2, and CRM56-2 showed the cholesterol assimilation of 46.59, 45.31, and 75.94 %, respectively. *L. brevis* strain CM38-1 showed the cholesterol assimilation of 62.54 %. *L. pentosus* strains CRM41-1, CRM41-2, NWM59-3, and NWM63-1 showed the cholesterol assimilation of 45.95, 69.56, 47.86, and 47.86 %, respectively. *L. paracasei* subsp. *tolerans* strains NWM61-1 and NWM62-1 showed the cholesterol assimilation

of 54.24 and 52.14 %, respectively. Furthermore, the other *Lactobacillus* sp. strains CRM40-3, CRM44-2, CRM47-3, CRM55-2, CRM56-1, and NWM59-1 showed the cholesterol assimilation of 65.73, 85.51, 61.26, 48.50, 55.52, and 46.76 %, respectively as shown in Table 4.9 and Figure 4.11. These LAB strains were selected to assess the probiotic properties as acid tolerance, bile tolerance, and adhesion assays. However, strains FM3-1, FM11-1, FM11-2, CM28-3, CM38-1, CRM41-1, CRM55-2, and NWM60-2 showed the cholesterol-lowering activities as BSH and cholesterol assimilation mechanisms.

Many *Lactobacillus* strains showed cholesterol assimilation such *as L. paracasei* and *L. pentosus*, and *L. plantarum* isolated from dairy products, *L. brevis* showed BSH activity but not showed cholesterol assimilation (Table 2.6) while *L. brevis* strain CM38-1 isolated from fermented tea leaves showed BSH activity and cholesterol assimilation for cholesterol-lowering. Moreover, *E. faecium* isolated from pickled turnip brine, stinky soybean, and kimchi while *E. faecium* strain FM11-1 isolated from flower that showed cholesterol assimilation and BSH activity for cholesterol-lowering (Xu *et al.,* 2016 ; Albano *et al.,* 2018).

Table 4.9 Cholesterol-lowering (BSH activity and cholesterol assimilation) of isolates

Strain no.	BSH	Cholesterol assimilation (%)
FM3-1	+	49.14 ± 0.002
FM11-1	+	46.59 ± 0.003
FM11-2	++	51.69 ± 0.007
CM28-3	+	46.59 ± 0.000
CM38-1	+	62.54 ± 0.006
CRM40-3	-	65.73 ± 0.004
CRM41-1	+	45.95 ± 0.001
CRM41-2	-	69.56 ± 0.005
CRM44-2	-	85.51 ± 0.008
CRM47-3	-	61.26 ± 0.003
CRM55-2	+	48.50 ± 0.008
CRM56-1	-	55.52 ± 0.006
CRM56-2	-	75.94 ± 0.003
NWM59-1	-	46.76 ± 0.005
NWM59-3	-	47.86 ± 0.006
NWM60-2	+	45.31 ± 0.003
NKM61-1	-	54.24 ± 0.004
NKM62-1	-	52.14 ± 0.007
NKM63-1	-	47.86 ± 0.006

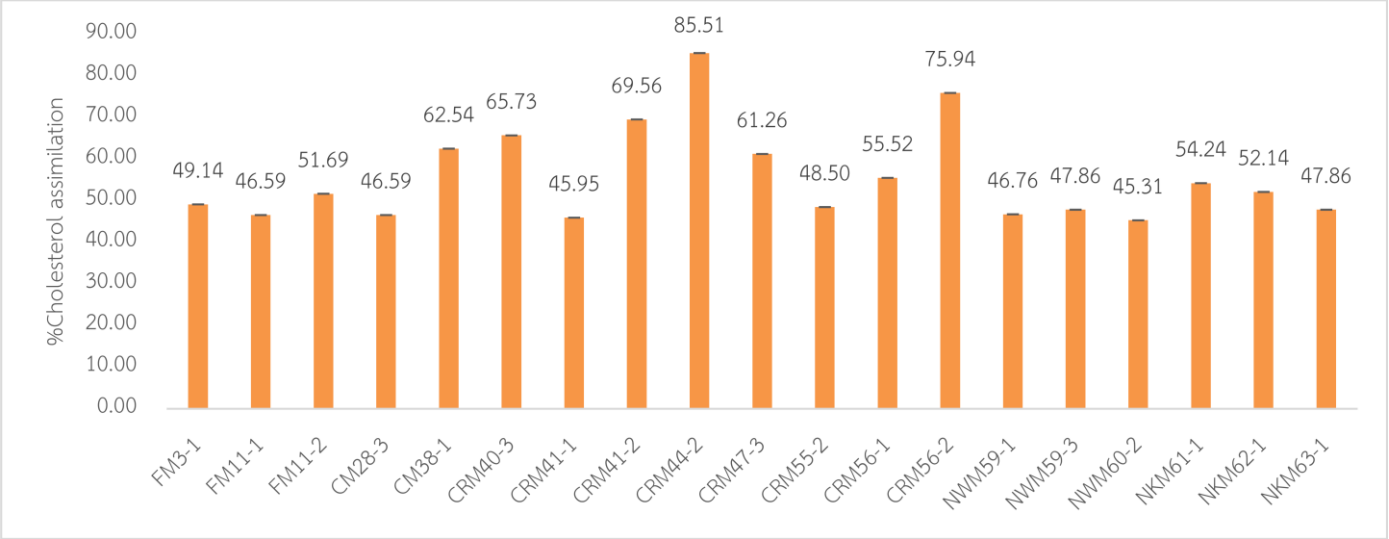

Figure 4.11 Cholesterol assimilation of nineteen strains in MRS broth supplemented 100 ug/ml of cholesterol, incubation (n = 3) for 48 h. MRS broth supplemented cholesterol without bacterial cells was used as control. Statistical analysis: ANOVA, Tukey's homogenous subsets generated from pair wise comparison. Significantly different compared to isolates ($P < 0.05$).

4.4.3 Antimicrobial activity

The supernatant (adjusted to pH 7.0) of *L. pentosus* strain CRM51-2 inoculum after incubated 48 h on MRS showed antimicrobial activity against *Bacillus subtil*is ATCC 6633 (9.1 mm) based on the agar well diffusion method. The other strains, excepted strain *Aerococcus urinaeequi* RYM18-2, did not show the antimicrobial activity against the tested microorganisms.

4.4.4 Cytotoxicity

Lactobacillus plantarum subsp. *plantarum* strain CM28-3 and *Pediococcus pentosaceus* strain CRM42-1 showed the cytotoxicity against Caco-2 cells as the cell viability of 64.0 $\pm$ 0.1 and 57.0 + 0.1 % while did not show the cytotoxicity against HepG2 and Vero cells based on MTT assay compared with cisplatin 50 μM and MRS as positive and negative control, respectively (Table 4.10). The other strains did not show cytotoxicity against Caco-2, HepG2 and Vero cells as the cut-off of 70.0 %.

Table 4.10 The percentage of cell viability of LAB based on MTT assay

Strain no.	Cell viability (% $\pm$ SD)		
	Vero	HepG2	Caco-2
CM28-3	257.0 + 0.2	100.0 $\pm$ 0.0	64.0 $\pm$ 0.1
CRM42-1	151.0 $\pm$ 0.7	100.0 $\pm$ 1.0	57.0 $\pm$ 0.1
Positive control	71.8 $\pm$ 0.1	18.9 $\pm$ 0.2	87.8 $\pm$ 0.0
Negative control	100.0 $\pm$ 0.4	100.0 $\pm$ 0.0	100.0 $\pm$ 0.0

4.4.5 Acid and bile tolerance

Thirty-one strains based on cholesterol lowering (BSH activity and cholesterol assimilation), antimicrobial activities, and cytotoxicity against cancer cells showed the resistance to acid and bile as the viability after acid (pH 3.0) and bile salt (0.3 %; pH 8.0) exposure for 0 and 3 hours. The survival of these strains after exposed to pH 3.0 and 0.3 % bile salts for 0 and 3 hours were shown as the viable counts (CFU/mL) in Table 4.11. The viability as viable counts (CFU/mL) of them showed various abilities of acid tolerance at pH 3.0 after incubation for 3 hours (as constant, increase, or decrease about 1 to 2 log cycles) while 0.3 % bile salt tolerance of them showed constant or increased about 1 log cycle of the viable counts after incubation for 3 hours as described in below.

Group A consisted of eight strains, including *Lactobacillus pentosus* strain CM27-1, CM33-1, and CRM41-1; *L. silagincola* strain CRM46-3; *L. plantarum* subsp. *plantarum* strain CRM56-2; *L. bevis* strain CM38-1; and *L. paracasei* subsp. *tolerans* strain NKM61-1 and NKM62-1 showed the acid tolerance at pH 3.0 as increasing about 1 log cycle of the viable counts after incubation 3 hours.

Group B consisted of fifteen strains, including *L. pentosus* strain FM14-1, FM14-2, F15-2, FM16-2, CM24-4, CM25-3, CM26-1, CM28-3, CRM39-1, CRM41-2, CRM51-2, NWM59-3, and NKM63-1; and *L. paracasei* subsp. *tolerans* strain NKM61-2 and NKM62-3 showed the acid tolerance at pH 3.0 as constant of the viable counts after incubation 3 hours.

Group C consisted of five strains, including *Enterococcus durans* strain FM1-1; *E. lactis* strain FM4-2; *L. plantarum* subsp. *plantarum* strain FM3-1 and FM15-1; and *P. acidilactici* strain FM9-2 showed the acid tolerance at pH 3.0 as decreasing about 1 log cycle of the viable counts after incubation 3 hours.

Group D consisted of six strains, including *E. durans* strain FM1-2 and FM12-1, *E. gallinarum* strain FM2-3, *Enterococcus* sp. strain FM11-1, *E. lactis* strain FM11-2, and *L. paracasei* subsp. *tolerans* strain NKM62-2 showed the acid tolerance at pH 3.0 as decreasing about 2 log cycles of the viable counts after incubation 3 hours.

Group A and B, as twenty-three strains, showed the potency of acid tolerance at pH 3.0. Therefore, group C (five strains) was declined during incubation at pH 3.0 the survived cells were remained about 5 log cycles after exposure for 3 hours as assigned the acid tolerance strains. Acid and bile tolerance is one of the most prior properties used to assess and select potentially probiotics that indicate ability to survive and colonize in the host small intestine (Hassanzadazar *et al.*, 2012)., accordingly thirty-four LAB strains were evaluated the other probiotic properties for capability and safety as the hemolytic type of *Enterococcus* sp. (nine strains) and adhesion assays. (Huang *et al.*, 2013) report that *L. plantarum* isolates exhibited high tolerance to low pH as well as high bile concentration with minimum cell count loss. These results revealed that *L. plantarumtum* Lp09 and Lp45 displayed an ability to tolerate acidic conditions and bile salts, thus, it could be a potential probiotic candidate.

Table 4.11 Survival of thirty-one LAB after incubated 0 and 3 h at pH 3 and 0.3 % bile salt

Strain no.	Acid pH 3 (CFU/mL)		Bile 0.3 %, pH 8 (CFU/mL)	
	0 h	3 h	0 h	3 h
FM1-1	3.00E+06	3.00E+05	7.00E+06	6.00E+06
FM1-2	5.00E+06	7.00E+04	6.00E+05	1.20E+06
FM2-3	1.20E+06	7.00E+04	1.50E+06	2.00E+06
FM3-1	4.00E+07	3.00E+06	6.00E+07	1.30E+08
FM4-2	1.90E+06	3.00E+05	2.70E+05	2.20E+06
FM9-2	7.33E+06	4.17E+05	1.60E+06	7.50E+06
FM11-1	4.67E+06	4.17E+04	3.83E+06	3.33E+06
FM11-2	5.00E+06	3.00E+04	4.00E+06	3.00E+06
FM12-1	5.00E+06	6.00E+04	1.80E+07	2.60E+07
FM14-1	3.00E+05	9.00E+05	5.00E+05	1.50E+06
FM14-2	3.00E+05	4.00E+05	7.00E+05	2.20E+06
FM15-1	3.00E+06	3.00E+05	1.00E+06	7.00E+06
FM15-2	3.00E+05	3.00E+05	3.00E+05	2.80E+06
FM16-2	5.00E+05	1.50E+05	5.00E+05	1.50E+06
CM24-4	1.70E+05	1.00E+05	1.80E+05	8.00E+06
CM25-3	1.10E+05	1.60E+05	2.00E+05	2.20E+06
CM26-1	7.00E+05	5.00E+05	9.00E+05	1.80E+06
CM27-1	8.00E+05	8.00E+06	1.00E+05	1.60E+05

Table 4.11 Survival of thirty-four LAB after incubated 0 and 3 h at pH 3 and 0.3 % bile salt (continued).

Strain no.	Acid pH 3 (CFU/mL)		Bile 0.3 %, pH 8 (CFU/mL)	
	0 h	3 h	0H	0 h
CM28-3	4.17E+06	5.17E+06	8.67E+06	1.40E+06
CM33-1	6.00E+05	3.00E+06	6.00E+05	8.00E+05
CM38-1	5.00E+06	1.20E+07	5.30E+06	1.20E+06
CRM39-1	5.17E+06	8.50E+06	5.33E+06	6.50E+06
CRM41-1	6.67E+06	2.25E+07	7.33E+06	1.20E+06
CRM41-2	1.03E+06	6.50E+06	5.17E+05	3.30E+06
CRM46-3	6.00E+05	6.00E+06	8.00E+06	7.00E+06
CRM51-2	6.33E+06	2.40E+06	8.70E+06	1.28E+06
CRM56-2	5.80E+06	2.40E+07	2.70E+06	7.67E+06
NWM59-3	1.53E+07	1.55E+07	8.33E+06	1.40E+06
NKM61-1	5.17E+06	2.30E+07	5.00E+06	9.67E+06
NKM62-1	7.67E+05	4.17E+06	1.30E+06	1.50E+06
NKM63-1	1.40E+06	4.83E+06	8.83E+06	5.00E+06

4.4.6 Adhesion assays

Thirteen strains of twenty-seven selected LAB strains (with *Enterococcus* sp. FM11-1 as candidate novel species and represented in *Entreococci*), based on acid tolerance, bile tolerance, nonhemolytic, and bioactivities, showed higher adhesion ability than the positive control as *L. rhamnosus* GG (0.40 ± 0.05 %) as shown in Table 4.12.

Table 4.12 The adhesion ability of 13 strains on Caco-2 cells.
L. rhamnosus strain GG was used as a positive control.

Strains no.	Adhesion ± SD (%)
FM3-1	0.40 ± 0.12
FM9-2	2.10 ± 0.10
FM11-1	0.43 ± 0.11
FM14-1	0.57 ± 0.12
CM28-3	0.70 + 0.17
CM38-1	6.00 ± 1.00
CRM39-1	0.67 ± 0.06
CRM41-2	0.50 ± 1.00
CRM56-2	0.40 ± 0.15
NWM59-3	4.20 ± 0.57
NKM61-1	0.40 ± 0.06
NKM62-1	0.50 ± 0.04
NKM63-1	2.80 ± 0.11
L. rhamnosus GG	0.40 ± 0.05

4.5 LAB strains as Probiotics

In this study, twelve LAB strains were candidate the probiotics with cholesterol-lowering that strain CM38-1 and CRM56-2 showed the highest potency. Almost of them belonged to the genus *Lactobacillus* (11 strains) and *Pediococcus* (1 strain) as shown in Table 4.13. *Enterococcus* sp. strain FM11-1 had alpha-hemolysis while *Lactobacillus* strain CRM42-1 and CRM51-2 did not resist to acid pH 3.0.

Table 4.13 The candidate strains of isolated LAB as probiotics

Genus	Strain no.	BSH	Cholesterol assimilation (%)	Cytotoxicity Caco-2	Antimicrobial	Tolerance		Adhesion ± SD (%)
						Acid pH3	Bile 0.3%	
L	FM3-1	+	49.14	-	-	+	+	0.40 ± 0.12
P	FM9-2	+	44.03	-	-	+	+	2.10 ± 0.10
E	FM11-1	+	46.59	-	-	+	+	0.43 ± 0.11
L	FM14-1	+	27.44	-	-	+	+	0.57 ± 0.12
L	CM28-3	+	46.59	+	-	+	+	0.70 + 0.17
L	CM38-1	+	62.54	-	-	+	+	6.00 ± 1.00
L	CRM39-1	+	34.46	-	-	+	+	0.67 ± 0.06
L	CRM41-2	+	69.56	-	-	+	+	0.50 ± 1.00
L	CRM42-1	-	35.74	+	-	-	+	ND
L	CRM51-2	-	39.91	-	+	-	+	ND
L	CRM56-2	-	75.94	-	-	+	+	0.40 ± 0.15
L	NWM59-	-	47.86	-	-	+	+	4.20 ± 0.57
L	NKM61-1	-	54.24	-	-	+	+	0.40 ± 0.06
L	NKM62-1	-	52.14	-	-	+	+	0.50 ± 0.04
L	NKM63-1	-	47.36	-	-	+	+	2.80 ± 0.11

L, *Lactobacillus*; E, *Enterococcus*; P, *Pediococcus*; ND, not determined

CHAPTER V

CONCLUSION

Seventy lactic acid bacteria (LAB) were isolated from nine flowers (17 strains), tree barks (7 strains), one fruit (2 strains), and fermented plants, ten tea leaves (31 strains) and two silage (13 strains). They were identified as *Lactobacillus pentosus* (12 strains), *L. plantarum* subsp. *plantarum* (9 strains), *L. paracasei* subsp. *tolerans* (5 strains), *L. brevis* (1 strain), *L. silagincola* (1 strain), *L. kunkeei* (1 strain), and *L. formosensis* (1 strain), *Enterococcus durans* (3 strains), *E. lactis* (2 strains), *E. faecalis* (1 strain), *E. faecium* (1 strain), *E. gallinarum* (1 strain), *E. gilvus* (1 strain), *Pediococcus acidilactici* (1 strain), *P. pentosaceus* (1 strain), and *Aerococcus urinaeequi* (1 strain) based on phenotypic characteristics and 16S rRNA gene sequence. The strain FM11-1^{T}, proposed to be *Enterococcus solani* sp. nov. was isolated from the flower of *Solanum torvum*, based on a polyphasic approach, including phenotypic, chemotaxonomic, and genotypic characteristics.

For the screening of probiotic activity including cholesterol-lowering, antimicrobial activity, and cytotoxicity, the results revealed that 12 strains, *L. plantarum* subsp. *plantarum* FM3-1, *L. plantarum* subsp. *plantarum* CM28-3, *L. brevis* CM38-1, and *L. pentosus* CRM41-2 showed BSH activity and cholesterol assimilation; moreover, *L. plantarum* subsp. *plantarum* CM28-3 showed the cytotoxicity against Caco-2 cells. *L. plantarum* subsp. *plantarum* CRM56-2,

L. pentosus NWM59-3, *L. paracasei* subsp. *tolerans* NKM61-1, *L. paracasei* subsp. *tolerans* NKM62-1, and *L. pentosus* NKM63-1 exhibited cholesterol assimilation. *L. pentosus* FM14-1 and *L. pentosus* CRM39-1 showed BSH activity. In addition, *P. acidilactici* FM9-2 exibited BSH activity and cholesterol assimilation. Furthermore, these strains should be studied the efficacy, safety, and other benefits in the *in vivo* testing before used as the probiotics. Although most of the probiotics belonged to *Lactobacillus* strains and *P. acidilactici* MTCC5101 producing bacteriocin was to report to use as the probiotic in human (Balgir *et al.,* 2013), *P. acidilactici* CNCM MA 18/5 M (Bactocell®) was used as the feed additive for shrimp, pig, and chicken (Castex, 2009).

In this study, a lot of lactic acid bacteria were isolated from plant samples and were found diverse species in the genera *Lactobacillus, Enterococcus, Aerococccus*, and *Pediococcus*. The plants and fermented plants are high potential resources of novel LAB species and new probiotics with many useful bioactivities.

APPENDIX A

CULTURE MEDIUM

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.

MRS medium

Glucose	20 g
Casein peptone, tryptic digest	10 g
Beef extract	10 g
Yeast extract	5 g
Sodium acetate	5 g
Dipotassium phosphate	2 g
Ammonium citrate	2 g
Tween 80	1 g
Magnesium Sulfate	0.2 g
Manganese sulfate	50 mg
Distilled water	1000 mL

BSH medium

MRS	5.5 g
Sodium salt of taurodeoxycholic acid	0.5 g
Calcium chloride	0.37 g
Distilled water	100 mL

Acid Production

Yeast extract	0.4 g
Peptone	0.5 g
Sugar	0.5 g
Salt solution	0.5 g
Distilled water	100 mL

Salt solution

$MgSO_4.7H_2O$	400 mg
$MnSO_4.5H_2O$	20 mg
$FeSO_4.7H_2O$	20 mg
NaCl	20 mg
Distilled water	10 mL

Arginine

Peptone	0.5 g
NaCl	0.5 g
Arginine	0.5 g
Agar	0.3 g
Yeast extract	0.1 g
K2HPO4	0.03 g
Phenol red	0.001 g
Distilled water	100 mL

Arginine hydrolysis was examined using arginine agar slant. The positive reaction is shown by a color change of the indicator to red after incubation for 5-7 days.

Nitrate reduction medium

Peptone	10 g
NaCl	10 g
KNO3	1 g
Yeast extract	0.3 g
Distilled water	100 mL
pH 6.8	

The ability to reduce nitrate to nitrite was determined using nitrate broth. After incubation, the culture broth was added with nitrate reduction test reagent. The pink to the red color represented the presence of nitrite (positive).

Aesculin

Beef extract	0.5 g
Yeast extract	0.5 g
Esculin	0.2 g
Glucose	0.25 g
Ferric Citrate	0.05 g
$MnSo_4$ $4H_2O$	0.01 g
Tween 80	0.1 g
Distilled water	100 m

APPENDIX B

REAGENTS AND BUFFERS

33% KOH

KOH	33 g
Distilled water	100 mL

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 7.2	

1X Tris-acetate (TAE) buffer

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

0.8% Agarose gel

Agarose	0.8 g
Distilled water	100 mL

Melt the mixture with the microwave.

0.85% NaCl

NaCl	0.85 g
Distilled water	100 mL

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 7.2

Nitrate Reagent A

Sulphanilic acid	0.8 gm
Acetic Acid, 5N	100 ml

Nitrate Reagent B

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

APPENDIX C

PCR REACTION

PCR master mixture	**Stock**	**(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 mM	2
$MgCl_2$	25 mM	8
Taq DNA polymerase	5 Unit/µL	0.5
Milli-Q water		66.5
Template	Undilute	5

PCR condition

Pre-denaturation	94 °C, 3 min	
Denaturation	94 °C, 1 min	30 cycle of PCR
Annealing	50 °C, 1 min	
Elongation	72 °C, 2 min	
Final elongation	72 °C, 3 min	
Hold	4 °C	

APPENDIX D

16S rRNA GENE SEQUENCES

>FM3-1 (1353 bp)

TTGCATCATGATTTACATTTGAGTGAGTGGCGAACTGGTGAGTAACACGTGGGAAACCTG
CCCAGAAGCGGGGGATAACACCTGGAAACAGATGCTAATACCGCATAACAACTTGGACCG
CATGGTCCGAGCTTGAAAGATGGCTTCGGCTATCACTTTTGGATGGTCCCGCGGCGTATT
AGCTAGATGGTGGGGTAACGGCTCACCATGGCAATGATACGTAGCCGACCTGAGAGGGTA
ATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAAT
CTTCCACAATGGACGAAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGGTTTCGGC
TCGTAAAACTCTGTTGTTAAAGAAGAACATATCTGAGAGTAACTGTTCAGGTATTGACGG
TATTTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGC
AAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTTTTAAGTCTGATGT
GAAAGCCTTCGGCTCAACCGAAGAAGTGCATCGGAAACTGGGAAACTTGAGTGCAGAAGA
GGACAGTGGAACTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAAGAACACCAGTGG
CGAAGGCGGCTGTCTGGTCTGTAACTGACGCTGAGGCTCGAAAGTATGGGTAGCAAACAG
GATTAGATACCCTGGTAGTCCATACCGTAAACGATGAATGCTAAGTGTTGGAGGGTTTCC
GCCCTTCAGTGCTGCAGCTAACGCATTAAGCATTCCGCCTGGGGAGTACGGCCGCAAGGC
TGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGA
AGCTACGCGAAGAACCTTACCAGGTCTTGACATACTATGCAAATCTAAGAGATTAGACGT
TCCCTTCGGGGACATGGATACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATG
TTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATTATCAGTTGCCAGCATTAAGTTGGGC
ACTCTGGTGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCAT
GCCCCTTATGACCTGGGCTACACACGTGCTACAATGGATGGTACAACGAGTTGCGAACTC
GCGAGAGTAAGCTAATCTCTTAAAGCCATTCTCAGTTCGGATTGTAGGCTGCAACTCGCC
TACATGAAGTCGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCG
GGCCTTGTACACACCGCCCGTCACACCATGAGA

>FM9-2 (1396bp)

TTCCGTTAATTGATTATGACGTGCTTGCACCGAATGAGATTTTAACACGAAGTGAGTGGC
GGACGGGTGAGTAACACGTGGGTAACCTGCCCAGAAGCAGGGGATAACACCTGGAAACAG
ATGCTAATACCGTATAACAGAGAAAACCGCCTGGTTTTCTTTTAAAAGATGGCTCTGCTA
TCACTTCTGGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGC
GATGATGCGTAGCCGACCTGAGAGGGTAATCGGCCACATTGGGACTGAGACACGGCCCAG
ACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGCAAGTCTGATGGAGCAA
CGCCGCGTGAGTGAAGAAGGGTTTCGGCTCGTAAAGCTCTGTTGTTAAAGAAGAACGTGG
GTGAGAGTAACTGTTCACCCAGTGACGGTATTTAACCAGAAAGCCACGGCTAACTACGTG
CCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGATTTATTGGGCGTAAAGCG
AGCGCAGGCGGTCTTTTAAGTCTAATGTGAAAGCCTTCGGCTCAACCGAAGAAGTGCATT
GGAAACTGGGAGACTTGAGTGCAGAAGAGGACAGTGGAACTCCATGTGTAGCGGTGAAAT
GCGTAGATATATGGAAGAACACCAGTGGCGAAGGCGGCTGTCTGGTCTGTAACTGACGCT
GAGGCTCGAAAGCATGGGTAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAAC
GATGATTACTAAGTGTTGGAGGGTTTCCGCCCTTCAGTGCTGCAGCTAACGCATTAAGTA
ATCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAAGAATTGACGGGGGCCCGCAC
AAGCGGTGGAGCATGTGGTTTAATTCGAAGCTACGCGAAGAACCTTACCAGGTCTTGACA
TCTTCTGCCAACCTAAGAGATTAGGCGTTCCCTTCGGGGACAGAATGACAGGTGGTGCAT
GGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTT
ATTACTAGTTGCCAGCATTCAGTTGGGCACTCTAGTGAGACTGCCGGTGACAAACCGGAG
GAAGGTGGGGACGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTAC
AATGGATGGTACAACGAGTCGCGAAACCGCGAGGTTTAGCTAATCTCTTAAAACCATTCT
CAGTTCGGACTGTAGGCTGCAACTCGCCTACACGAAGTCGGAATCGCTAGTAATCGCGGA
TCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGAG
AGTTTGTAACACCCAA

>FM14-1 (1396 bp)

CCTGGTATTGATTGGTGCTTGCATCATGATTTACATTTGAGTGAGTGGCGAACTGGTGAG
TAACACGTGGGAAACCTGCCCAGAAGCGGGGGATAACACCTGGAAACAGATGCTAATACC
GCATAACAACTTGGACCGCATGGTCCGAGTTTGAAAGATGGCTTCGGCTATCACTTTTGG
ATGGTCCCGCGGCGTATTAGCTAGATGGTGGGGTAACGGCTCACCATGGCAATGATACGT
AGCCGACCTGAGAGGGTAATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGG
GAGGCAGCAGTAGGGAATCTTCCACAATGGACGAAAGTCTGATGGAGCAACGCCGCGTGA
GTGAAGAAGGGTTTCGGCTCGTAAAACTCTGTTGTTAAAGAAGAACATATCTGAGAGTAA
CTGTTCAGGTATTGACGGTATTTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCG
CGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCG
GTTTTTTAAGTCTGATGTGAAAGCCTTCGGCTCAACCGAAGAAGTGCATCGGAAACTGGG
AAACTTGAGTGCAGAAGAGGACAGTGGAACTCCATGTGTAGCGGTGAAATGCGTAGATAT
ATGGAAGAACACCAGTGGCGAAGGCGGCTGTCTGGTCTGTAACTGACGCTGAGGCTCGAA
AGTATGGGTAGCAAACAGGATTAGATACCCTGGTAGTCCATACCGTAAACGATGAATGCT
AAGTGTTGGAGGGTTTCCGCCCTTCAGTGCTGCAGCTAACGCATTAAGCATTCCGCCTGG
GGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGA
GCATGTGGTTTAATTCGAAGCTACGCGAAGAACCTTACCAGGTCTTGACATACTATGCAA
ATCTAAGAGATTAGACGTTCCCTTCGGGGACATGGATACAGGTGGTGCATGGTTGTCGTC
AGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATTATCAGTT
GCCAGCATTAAGTTGGGCACTCTGGTGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGG
ATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGATGGT
ACAACGAGTTGCGAACTCGCGAGAGTAAGCTAATCTCTTAAAGCCATTCTCAGTTCGGAT
TGTAGGCTGCAACTCGCCTACATGAAGTCGGAATCGCTAGTAATCGCGGATCAGCATGCC
GCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGAGAGTTTGTAAC
ACCCAAAGTCGGTGGG

>CM28-3 (1397 bp)

TGGTATTGATTGGTGCTTGCATCATGATTTACATTTGAGTGAGTGGCGAACTGGTGAGTA
ACACGTGGGAAACCTGCCCAGAAGCGGGGGATAACACCTGGAAACAGATGCTAATACCGC
ATAACAACTTGGACCGCATGGTCCGAGCTTGAAAGATGGCTTCGGCTATCACTTTTGGAT
GGTCCCGCGGCGTATTAGCTAGATGGTGGGGTAACGGCTCACCATGGCAATGATACGTAG
CCGACCTGAGAGGGTAATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGA
GGCAGCAGTAGGGAATCTTCCACAATGGACGAAAGTCTGATGGAGCAACGCCGCGTGAGT
GAAGAAGGGTTTCGGCTCGTAAAACTCTGTTGTTAAAGAAGAACATATCTGAGAGTAACT
GTTCAGGTATTGACGGTATTTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCG
GTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGT
TTTTTAAGTCTGATGTGAAAGCCTTCGGCTCAACCGAAGAAGTGCATCGGAAACTGGGAA
ACTTGAGTGCAGAAGAGGACAGTGGAACTCCATGTGTAGCGGTGAAATGCGTAGATATAT
GGAAGAACACCAGTGGCGAAGGCGGCTGTCTGGTCTGTAACTGACGCTGAGGCTCGAAAG
TATGGGTAGCAAACAGGATTAGATACCCTGGTAGTCCATACCGTAAACGATGAATGCTAA
GTGTTGGAGGGTTTCCGCCCTTCAGTGCTGCAGCTAACGCATTAAGCATTCCGCCTGGGG
AGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGC
ATGTGGTTTAATTCGAAGCTACGCGAAGAACCTTACCAGGTCTTGACATACTATGCAAAT
CTAAGAGATTAGACGTTCCCTTCGGGGACATGGATACAGGTGGTGCATGGTTGTCGTCAG
CTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATTATCAGTTGC
CAGCATTAAGTTGGGCACTCTGGTGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGAT
GACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGATGGTAC
AACGAGTTGCGAACTCGCGAGAGTAAGCTAATCTCTTAAAGCCATTCTCAGTTCGGATTG
TAGGCTGCAACTCGCCTACATGAAGTCGGAATCGCTAGTAATCGCGGATCAGCATGCCGC
GGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGAGAGTTTGTAACAC
CCAAAGTCGGTGGGGTA

>CM38-1 (1379 bp)

TTGCACTGATTTCAACAATGAAGCGAGTGGCGAACTGGTGAGTAACACGTGGGAAATCTG
CCCAGAAGCAGGGGATAACACTTGGAAACAGGTGCTAATACCGTATAACAACAAAATCCG
CATGGATTTTGTTTGAAAGGTGGCTTCGGCTATCACTTCTGGATGATCCCGCGGCGTATT
AGTTAGTTGGTGAGGTAAAGGCCCACCAAGACGATGATACGTAGCCGACCTGAGAGGGTA
ATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAAT
CTTCCACAATGGACGAAAGTCTGATGGAGCAATGCCGCGTGAGTGAAGAAGGGTTTCGGC
TCGTAAAACTCTGTTGTTAAAGAAGAACACCTTTGAGAGTAACTGTTCAAGGGTTGACGG
TATTTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGC
AAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTTTTAAGTCTGATGT
GAAAGCCTTCGGCTTAACCGGAGAAGTGCATCGGAAACTGGGAGACTTGAGTGCAGAAGA
GGACAGTGGAACTCCATGTGTAGCGGTGGAATGCGTAGATATATGGAAGAACACCAGTGG
CGAAGGCGGCTGTCTAGTCTGTAACTGACGCTGAGGCTCGAAAGCATGGGTAGCGAACAG
GATTAGATACCCTGGTAGTCCATGCCGTAAACGATGAGTGCTAAGTGTTGGAGGGTTTCC
GCCCTTCAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGT
TGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGA
AGCTACGCGAAGAACCTTACCAGGTCTTGACATCTTCTGCCAATCTTAGAGATAAGACGT
TCCCTTCGGGGACAGAATGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATG
TTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATTATCAGTTGCCAGCATTCAGTTGGGC
ACTCTGGTGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCAT
GCCCCTTATGACCTGGGCTACACACGTGCTACAATGGACGGTACAACGAGTTGCGAAGTC
GTGAGGCTAAGCTAATCTCTTAAAGCCGTTCTCAGTTCGGATTGTAGGCTGCAACTCGCC
TACATGAAGTTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCG
GGCCTTGTACACACCGCCCGTCACACCATGAGAGTTTGTAACACCCAAAGCCGGTGAGA

>CRM39-1 (1422 bp)

GAACGAACTCTGGTATTGATTGGTGCTTGCATCATGATTTACATTTGAGTGAGTGGCGAA
CTGGTGAGTAACACGTGGGAAACCTGCCCAGAAGCGGGGGATAACACCTGGAAACAGATG
CTAATACCGCATAACAACTTGGACCGCATGGTCCGAGTTTGAAAGATGGCTTCGGCTATC
ACTTTTGGATGGTCCCGCGGCGTATTAGCTAGATGGTGGGGTAACGGCTCACCATGGCAA
TGATACGTAGCCGACCTGAGAGGGTAATCGGCCACATTGGGACTGAGACACGGCCCAAAC
TCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGAAAGTCTGATGGAGCAACG
CCGCGTGAGTGAAGAAGGGTTTCGGCTCGTAAAACTCTGTTGTTAAAGAAGAACATATCT
GAGAGTAACTGTTCAGGTATTGACGGTATTTAACCAGAAAGCCACGGCTAACTACGTGCC
AGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAG
CGCAGGCGGTTTTTTAAGTCTGATGTGAAAGCCTTCGGCTCAACCGAAGAAGTGCATCGG
AAACTGGGAAACTTGAGTGCAGAAGAGGACAGTGGAACTCCATGTGTAGCGGTGAAATGC
GTAGATATATGGAAGAACACCAGTGGCGAAGGCGGCTGTCTGGTCTGTAACTGACGCTGA
GGCTCGAAAGTATGGGTAGCAAACAGGATTAGATACCCTGGTAGTCCATACCGTAAACGA
TGAATGCTAAGTGTTGGAGGGTTTCCGCCCTTCAGTGCTGCAGCTAACGCATTAAGCATT
CCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAA
GCGGTGGAGCATGTGGTTTAATTCGAAGCTACGCGAAGAACCTTACCAGGTCTTGACATA
CTATGCAAATCTAAGAGATTAGACGTTCCCTTCGGGGACATGGATACAGGTGGTGCATGG
TTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTAT
TATCAGTTGCCAGCATTAAGTTGGGCACTCTGGTGAGACTGCCGGTGACAAACCGGAGGA
AGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAA
TGGATGGTACAACGAGTTGCGAACTCGCGAGAGTAAGCTAATCTCTTAAAGCCATTCTCA
GTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGTCGGAATCGCTAGTAATCGCGGATC
AGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGAGAG
TTTGTAACACCCAAAGTCGGTGGGGTAACCTTTTAGGAACCA

>CRM41-2 (1490 bp)

CACCCTAATCATCTGTCCCACCTTAGGCGGCTGGTTCCTAAAAGGTTACCCCACCGACTT
TGGGTGTTACAAACTCTCATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTC
ACCGCGGCATGCTGATCCGCGATTACTAGCGATTCCGACTTCATGTAGGCGAGTTGCAGC
CTACAATCCGAACTGAGAATGGCTTTAAGAGATTAGCTTACTCTCGCGAGTTCGCAACTC
GTTGTACCATCCATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGAC
GTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCTCACCAGAGTGCCCAACTTAATG
CTGGCAACTGATAATAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACAC
GAGCTGACGACAACCATGCACCACCTGTATCCATGTCCCCGAAGGGAACGTCTAATCTCT
TAGATTTGCATAGTATGTCAAGACCTGGTAAGGTTCTTCGCGTAGCTTCGAATTAAACCA
CATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCAGCCTTGCGGCCGT
ACTCCCCAGGCGGAATGCTTAATGCGTTAGCTGCAGCACTGAAGGGCGGAAACCCTCCAA
CACTTAGCATTCATCGTT-TACGGTATGGA-CTACC-AGGGTATCTAATCCTGTTTGCTA
CCCATACTTTCGAGCCTCAGC-GTCAGTTACAGACCAGACAGCCGCCTTCGCCACTGG-T
GTTCTTCCATATATCTACGCATTTCACCGCTACACATGGAGTTCCACTGTCCTCTTCTGC
ACTCAAGTTTCCCAGTTTCCGATGCACTTCTTCGGTTGAGCCGAAGGCTTTCACATCAGA
CTTAAAAAACCGCCTGCGCTCGCTTTACGCCCAATAAATCCGGACAACGCTTGCCACCTA
CGTATTACCGCGGCTGCTGGCACGTAGTT-AGCCGTGGCTTTCTGGTTAAATACCGTCAA
TACCTGAACAGTTACTCTCAGATATGTTCTTCTTTAACAACAGAGTTTTACGAGCCGAAA
CCCTTCTTCACTCACGCGGCGTTGCTCCATCAGACTTTCGTCCATTGTGGAAGATTCCCT
ACTGCTGCCTCCCGTAGGAGTTTGGGCCGTGTCTCAGTCCCAATGTGGCCGATTACCCTC
TCAGGTCGGCTACGTATCATTGCCATGGTGAGCCGTTACCCCACCATCTAGCTAATACGC
CGCGGGACCATCCAAAAGTGATAGCCGAAGCCATCTTTCAAACTCGGACCATGCGGTCCA
AGTTGTTATGCGGTATTAGCATCTGTTTCCAGGTGTTATCCCCCGCTTCTGGGCAGGTTT
CCCACGTGTTACTCACCAGTTCGCCACTCACTCAAATGTAAATCATGATGCAAGCACCAA
TCAATACCAGAGTTCGTTCGACTTGCATGTATTAGGCACGCCGCCAGCGTTCGTCT

>CRM42-1 (1441 bp)

GTCGAACGAACTTCCGTTAATTGATTATGACGTACTTGTACTGATTGAGATTTTAACACG
AAGTGAGTGGCGAACGGGTGAGTAACACGTGGGTAACCTGCCCAGAAGTAGGGGATAACA
CCTGGAAACAGATGCTAATACCGTATAACAGAGAAAACCGCATGGTTTTCTTTTAAAAGA
TGGCTCTGCTATCACTTCTGGATGGACCCGCGGCGTATTAGCTAGTTGGTGAGGTAAAGG
CTCACCAAGGCAGTGATACGTAGCCGACCTGAGAGGGTAATCGGCCACATTGGGACTGAG
ACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGCAAGTC
TGATGGAGCAACGCCGCGTGAGTGAAGAAGGGTTTCGGCTCGTAAAGCTCTGTTGTTAAA
GAAGAACGTGGGTAAGAGTAACTGTTTACCCAGTGACGGTATTTAACCCAGAAAGCCACG
GCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGATTTATT
GGGCGTAAAGCGAGCGCAGGCGGTCTTTTAAGTCTAATGTGAAAGCCTTCGGCTCAACCG
AAGAAGTGCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGACAGTGGAACTCCATGTG
TAGCGGTGAAATGCGTAGATATATGGAAGAACACCAGTGGCGAAGGCGGCTGTCTGGTCT
GCAACTGACGCTGAGGCTCGAAAGCATGGGTAGCGAACAGGATTAGATACCCTGGTAGTC
CATGCCGTAAACGATGATTACTAAGTGTTGGAGGGTTTCCGCCCTTCAGTGCTGCAGCTA
ACGCATTAAGTAATCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAAGAATTGAC
GGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCTACGCGAAGAACCTTAC
CAGGTCTTGACATCTTCTGACAGTCTAAGAGATTAGAGGTTCCCTTCGGGGACAGAATGA
CAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACG
AGCGCAACCCTTATTACTAGTTGCCAGCATTAAGTTGGGCACTCTAGTGAGACTGCCGGT
GACAAACCGGAGGAAGGTGGGGACGACGTCAAATCATCATGCCCCTTATGACCTGGGCTA
CACACGTGCTACAATGGATGGTACAACGAGTCGCGAGACCGCGAGGTTAAGCTAATCTCT
TAAAACCATTCTCAGTTCGGACTGTAGGCTGCAACTCGCCTACACGAAGTCGGAATCGCT
AGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCG
TCACACCATGAGAGTTTGTAACACCCAAAGCCGGTGGGGTAACCTTTTAGGAGCTAGCCG
T

>NWM59-3 (1396 bp)

CTCTGGTATTGATTGGTGCTTGCATCATGATTTACATTTGAGTGAGTGGCGAACTGGTGA
GTAACACGTGGGAAACCTGCCCAGAAGCGGGGGATAACACCTGGAAACAGATGCTAATAC
CGCATAACAACTTGGACCGCATGGTCCGAGTTTGAAAGATGGCTTCGGCTATCACTTTTG
GATGGTCCCGCGGCGTATTAGCTAGATGGTGGGGTAACGGCTCACCATGGCAATGATACG
TAGCCGACCTGAGAGGGTAATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACG
GGAGGCAGCAGTAGGGAATCTTCCACAATGGACGAAAGTCTGATGGAGCAACGCCGCGTG
AGTGAAGAAGGGTTTCGGCTCGTAAAACTCTGTTGTTAAAGAAGAACATATCTGAGAGTA
ACTGTTCAGGTATTGACGGTATTTAACCAGAAAGCCACGGCTAAATTACGTGCCAGCAGC
CGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGG
CGGTTTTTTAAGTCTGATGTGAAAGCCTTCGGCTCAACCGAAGAAGTGCATCGGAAACTG
GGAAACTTGAGTGCAGAAGAGGACAGTGGAACTCCATGTGTAGCGGTGAAATGCGTAGAT
ATATGGAAGAACACCAGTGGCGAAGGCGGCTGTCTGGTCTGTAACTGACGCTGAGGCTCG
AAAGTATGGGTAGCAAACAGGATTAGATACCCTGGTAGTCCATACCGTAAACGATGAATG
CTAAGTGTTGGAGGGTTTCCGCCCTTCAGTGCTGCAGCTAACGCATTAAGCATTCCGCCT
GGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTG
GAGCATGTGGGTTTAATTCGAAGCTACGCGAAGAACCTTACCAGGTCTTGACATACTATG
CAAATCTAAGAGATTAGACGTTCCCTTCGGGGACATGGATACAGGTGGTGCATGGTTGTC
GTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATTATCA
GTTGCCAGCATTAAGTTGGGCACTCTGGTGAGACTGCCGGTGACAAACCGGAGGAAGGTG
GGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGAT
GGTACAACGAGTTGCGAACTCGCGAGAGTAAGCTAATCTCTTAAAGCCATTCTCAGTTCG
GATTGTAGGCTGCAACTCGCCTACATGAAGTCGGAATCGCTAGTAATCGCGGATCAGCAT
GCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGAGAGTTTGT
AACACCCAAAGTCGGT

>NKM61-1 (1410 bp)

CTCGTTGATGATCGGTGCTTGCACCGAGATTCAACATGGAACGAGTGGCGGACGGGTGAG
TAACACGTGGGTAACCTGCCCTTAAGTGGGGGATAACATTTGGAAACAGATGCTAATACC
GCATAGATCCAAGAACCGCATGGTTCTTGGCTGAAAGATGGCGTAAGCTATCGCTTTTGG
ATGGACCCGCGGCGTATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGATGATACGT
AGCCGAACTGAGAGGTTGATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGG
GAGGCAGCAGTAGGGAATCTTCCACAATGGACGCAAGTCTGATGGAGCAACGCCGCGTGA
GTGAAGAAGGCTTTCGGGTCGTAAAACTCTGTTGTTGGAGAAGAATGGTCGGCAGAGTAA
CTGTTGTCGGCGTGACGGTATCCAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCG
CGGTAATACGTAGGTGGCAAGCGTTATCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCG
GTCTAGTAAGTCTGATGAGAAAGCCCTCGGCTTAACCGAGGAAGCGCATCGCAAACTGGG
AAACTTGAGTGCAGAAGAGGACAGTGGAACTCCATGTGTAGCGGTGAAATGCGTAGATAT
ATGGAAGAACACCAGTGGCGAAGGCGGCTGTCTGGTCTGTAACTGACGCTGAGGCTCGAA
AGCATGGGTAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGATGAATGCT
AGGTGTTGGAGGGTTTCCGCCCTTCAGTGCCGCAGCTAACGCATTAAGCATTCCGCCTGG
GGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGA
GCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCTTTTGATC
ACCTGAGAGATCAGGTTTCCCCTTCGGGGGCAAAATGACAGGTGGTGCATGGTTGTCGTC
AGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATGACTAGTT
GCCAGCATTTAGTTGGGCACTCTAGTAAGACTGCCGGTGACAAACCGGAGGAAGGTGGGG
ATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGATGGT
ACAACGAGTTGCGAGACCGCGAGGTCAAGCTAATCTCTTAAAGCCATTCTCAGTTCGGAC
TGTAGGCTGCAACTCGCCTACACGAAGTCGGAATCGCTAGTAATCGCGGATCAGCACGCC
GCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGAGAGTTTGTAAC
ACCCGAAGCCGGTGGCGTAACCCTTTTAGG

>NKM62-1 (1394 bp)

CTCGTTGATGATCGGTGCTTGCACCGAGATTCAACATGGAACGAGTGGCGGACGGGTGAG
TAACACGTGGGTAACCTGCCCTTAAGTGGGGGATAACATTTGGAAACAGATGCTAATACC
GCATAGATCCAAGAACCGCATGGTTCTTGGCTGAAAGATGGCGTAAGCTATCGCTTTTGG
ATGGACCCGCGGCGTATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGATGATACGT
AGCCGAACTGAGAGGTTGATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGG
GAGGCAGCAGTAGGGAATCTTCCACAATGGACGCAAGTCTGATGGAGCAACGCCGCGTGA
GTGAAGAAGGCTTTCGGGTCGTAAAACTCTGTTGTTGGAGAAGAATGGTCGGCAGAGTAA
CTGTTGCCGGCGTGACGGTATCCAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCG
CGGTAATACGTAGGTGGCAAGCGTTATCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCG
GTTTTTTAAGTCTGATGTGAAAGCCCTCGGCTTAACCGAGGAAGCGCATCGGAAACTGGG
AAACTTGAGTGCAGAAGAGGACAGTGGAACTCCATGTGTAGCGGTGAAATGCGTAGATAT
ATGGAAGAACACCAGTGGCGAAGGCGGCTGTCTGGTCTGTAACTGACGCTGAGGCTCGAA
AGCATGGGTAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGATGAATGCT
AGGTGTTGGAGGGTTTCCGCCCTTCAGTGCCGCAGCTAACGCATTAAGCATTCCGCCTGG
GGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGA
GCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCTTTTGATC
ACCTGAGAGATCAGGTTTCCCCTTCGGGGGCAAAATGACAGGTGGTGCATGGTTGTCGTC
AGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATGACTAGTT
GCCAGCATTTAGTTGGGCACTCTAGTAAGACTGCCGGTGACAAACCGGAGGAAGGTGGGG
ATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGATGGT
ACAACGAGTTGCGAGACCGCGAGGTCAAGCTAATCTCTTAAAGCCATTCTCAGTTCGGAC
TGTAGGCTGCAACTCGCCTACACGAAGTCGGAATCGCTAGTAATCGCGGATCAGCACGCC
GCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGAGAGTTTGTAAC
ACCCGAAGCCGGTG

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