สมบัติโปรไบโอติกและฤทธิ์ต้าน *Helicobacter pylori* ของแบคทีเรียกรดแลกติกจากผลิตภัณฑ์พืชและอาหารหมัก

นางสุจิตรา เตโช

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PROBIOTIC PROPERTIES AND ANTI-*HELICOBACTER PYLORI* ACTIVITY OF LACTIC ACID BACTERIA FROM PLANT AND FERMENTED FOOD PRODUCTS

Mrs. Sujitra Techo

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A Dissertation Submitted in Partial Fulfillment of the Requirements
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สุจิตรา เตโช : สมบัติโปรไบโอติกและฤทธิ์ต้าน *Helicobacter pylori* ของแบคทีเรียกรดแลกติกจากผลิตภัณฑ์พืชและอาหารหมัก (PROBIOTIC PROPERTIES AND ANTI-*HELICOBACTER PYLORI* ACTIVITY OF LACTIC ACID BACTERIA FROM PLANT AND FERMENTED FOOD PRODUCTS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ศ. ดร.สมบูรณ์ ธนาศุภวัฒน์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ดร.วรรณพ วิเศษสงวน, 121 หน้า.

แบคทีเรียกรดแลกติกทั้งหมด 238 สายพันธุ์ที่คัดแยกจากผลิตภัณฑ์พืชและอาหารหมักหลายชนิดในประเทศไทยถูกคัดกรองฤทธิ์ต้านแบคทีเรีย คัดเลือกแบคทีเรีย 53 สายพันธุ์ที่มีฤทธิ์ต้าน *Helicobacter pylori* และพิสูจน์เอกลักษณ์โดยอาศัยลักษณะทางฟีโนไทป์ อนุกรมวิธานเคมี และการวิเคราะห์ลำดับเบสช่วงยีน 16S rRNA สามารถพิสูจน์เอกลักษณ์ได้เป็น *Lactobacillus* (*Lb.*) *plantarum* subsp. *plantarum* (3 สายพันธุ์), *Lb. pentosus* (10 สายพันธุ์), *Pediococcus* (*P.*) *pentosaceus* (2 สายพันธุ์), *Lb. paracasei* subsp. *tolerans* (1 สายพันธุ์), *Lb. fermentum* (5 สายพันธุ์), *Enterococcus* (En.) *faecium* (2 สายพันธุ์), *En. casseliflavus* (1 สายพันธุ์), *Lactococcus* (*Lc.*) *garvieae* subsp. *garvieae* (1 สายพันธุ์), *Lc. lactis* subsp. *lactis* (3 สายพันธุ์), *Leuconostoc* (*Ln.*) *lactis* (2 สายพันธุ์), *Ln. citreum* (2 สายพันธุ์), *Ln. pseudomesenteroides* (2 สายพันธุ์), *Weissella* (*W.*) *paramesenteroides* (1 สายพันธุ์), *W. cibaria* (6 สายพันธุ์) และ *W. confusa* (11 สายพันธุ์) สายพันธุ์ Ru20-1^{T} ที่แยกจากดอกเข็ม (*Ixora coccinea* L.) มีความใกล้เคียงกับ *Lb. lindneri* LMG 14528^{T} (96.8 %) และ *Lb. sanfranciscensis* NRIC 1548^{T} (92.0 %) เมื่อวิเคราะห์ความคล้ายคลึงของลำดับเบสช่วงยีน 16S rRNA และ *pheS* ตามลำดับ การวิเคราะห์แผนภาพวิวัฒนาการบ่งชี้ว่าสายพันธุ์ Ru20-1^{T} แยกออกจากสปีชีส์อื่นในสกุล *Lactobacillus* อย่างชัดเจน ดังนั้นสายพันธุ์ Ru20-1^{T} จึงถูกเสนอเป็นแบคทีเรียสปีชีส์ใหม่ชื่อว่า *Lactobacillus ixorae*

การวิเคราะห์ความสามารถในการเกาะติดของสายพันธุ์ที่คัดเลือกบน AGS cell lines พบว่า *Ln. pseudomesenteroides* สายพันธุ์ BGM-S4 มีความสามารถในการเกาะติดสูงสุด (20.4%) ลำดับถัดมาคือ *Ln. pseudomesenteroides* สายพันธุ์ FM-S16 (12.9%) ในขณะที่สายพันธุ์อื่นมีความสามารถในการเกาะติดต่ำกว่า 5% เนื่องจาก *Lb. fermentum* P43-01 มีฤทธิ์ต้าน *H. pylori* MS83 และ BK364 จึงถูกคัดเลือกเพื่อประเมินความสามารถในการทนกรดและกรดน้ำดี สายพันธุ์นี้มีการรอดชีวิตคงที่เมื่อทดสอบในสภาวะพีเอช 3 (7.6 log10 cfu/ml) และมีจำนวนลดลง 2 log cycles เมื่อทดสอบในสภาวะพีเอช 2 นาน 3 ชั่วโมง หลังจากบ่มในสภาวะกรดน้ำดี 0.3% และ 0.5% นาน 6 ชั่วโมง แบคทีเรียมีจำนวนเพิ่มขึ้น 1.5 และ 1 log cycles ตามลำดับ

การย่อยสารประเภทโปรตีนที่ผลิตจากสายพันธุ์ FM-S16 และสายพันธุ์ P43-01 ด้วยโปรตีเอส พบว่า BLS ของสายพันธุ์ FM-S16 ไวต่อโปรตีเนสเค ขณะที่ BLS ของสายพันธุ์ P43-01 ไวต่ออัลฟา-ไคโมทริปซิน และเปปซิน ผลการทดลองนี้แสดงให้เห็นว่า BLS จากสายพันธุ์ที่คัดเลือกน่าจะเป็นกลไกสำคัญที่ยับยั้ง *H. pylori* เนื่องจากสายพันธุ์ FM-S16 มีฤทธิ์ต้าน *H. pylori* สูงสุด จึงคัดเลือก BLS ของสายพันธุ์ดังกล่าวนี้ไปทำให้บริสุทธิ์ผ่านเทคนิค hydrophobic interaction, cation-exchange และ reverse-phase high performance liquid chromatography ทำให้ได้ความบริสุทธิ์เพิ่มขึ้น 380 เท่า และมีน้ำหนักโมเลกุล 1094-1316 ดาลตัน

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สาขาวิชา	เภสัชเคมีและผลิตภัณฑ์ธรรมชาติ	ลายมือชื่อ อ.ที่ปรึกษาหลัก	.....
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SUJITRA TECHO: PROBIOTIC PROPERTIES AND ANTI-*HELICOBACTER PYLORI* ACTIVITY OF LACTIC ACID BACTERIA FROM PLANT AND FERMENTED FOOD PRODUCTS. ADVISOR: PROF. SOMBOON TANASUPAWAT, Ph.D., CO-ADVISOR: WONNOP VISSESSANGUAN, Ph.D., 121 pp.

Two hundred and thirty-eight strains of lactic acid bacteria (LAB) isolated from several plant-materials and fermented food products collected in Thailand were screening for antibacterial activity. Fifty-three strains which exhibited antimicrobial activity against *Helicobacter pylori* clinical strains, were selected and identified based on the phenotypic, chemotaxonomic characteristics and 16S rRNA gene sequence analysis. They were identified as *Lactobacillus* (*Lb.*) *plantarum* subsp. *plantarum* (3 strains), *Lb. pentosus* (10 starins), *Pediococcus* (*P.*) *pentosaceus* (2 strains), *Lb. paracasei* subsp. *tolerans* (1 strain), *Lb. fermentum* (5 strains), *Enterococcus* (*En.*) *faecium* (2 strains), *En. casseliflavus* (1 strain), *Lactococcus* (*Lc.*) *garvieae* subsp. *garvieae* (1 strain), *Lc. lactis* subsp. *lactis* (3 strains), *Leuconostoc* (*Ln.*) *lactis* (2 strains), *Ln. citreum* (2 strains), *Ln. pseudomesenteroides* (2 strains), *Weissella* (*W.*) *paramesenteroides* (1 strain), *W. cibaria* (6 strains) and *W. confusa* (11 strains). Strain Ru20-1^T isolated from West-Indian jasmine (*Ixora coccinea* L.) was closely related to *Lb. lindneri* LMG 14528^T (96.8 %) and *Lb. sanfranciscensis* NRIC 1548^T (92.0 %) based on 16S rRNA gene sequence and *pheS* gene sequence similarity, respectively. Phylogenetic analysis indicated that strain Ru20-1^T was clearly separated from related species of the genus *Lactobacillus*. On the basis of polyphasic taxonomy, strain Ru20-1^T was proposed as *Lactobacillus ixorae* sp. nov.

The adherence abilities assay of selected strains on AGS cell lines, *Ln. pseudomesenteroides* strain BGM-S4 showed the highest adhesion ability (20.4%) followed by *Ln. pseudomesenteroides* strain FM-S16 (12.9%). While the other strains exhibited the adherence lower than 5%. *Lb. fermentum* P43-01 exhibited antimicrobial activity against *H. pylori* MS83 and BK364, was selected for evaluate the acid and bile tolerance. This strain had constant viability at pH 3 (7.6 log10 cfu/ml) and the numbers were approximately decreased 2 log cycles after incubation at pH 2 for 3 h. After exposure to bile acids for 6 h at 0.3% and 0.5%, a strain was slightly increased 1.5 and 1 log cycles, respectively.

The treatment of proteinaceous compounds, bacteriocin-like substances (BLS), produced by strain FM-S16 and P43-01 using protease, revealed that BLS of strain FM-S16 was sensitive to proteinase K while the BLS of strain P43-01 was sensitive to a-chymotrypsin and pepsin. These indicated that BLS of the selected strains might be the significant mechanism that exerted the inhibition on *H. pylori*. According to the highest anti-*H. pylori* activity of strain FM-S16, it was chosen for partial purification through the hydrophobic interaction, cation-exchange and reverse-phase high performance liquid chromatography with 380-fold of purity. The molecular mass of partially purified bacteriocin was in the range of 1094-1316 Da.

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Department:	Food and Pharmaceutical Chemistry	Student's Signature .....
Field of Study:	Pharmaceutical Chemistry and Natural Products	Advisor's Signature .....
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CHAPTER 1
INTRODUCTION

Helicobacter pylori was successfully cultivated by Warren and Marshall in 1983 who first described this bacterium is associated with chronic gastritis and peptic ulcer (Warren and Marshall, 1983). *H. pylori* commonly colonized on the mucous layer of the gastric epithelium of the human stomach. Hence, it has been isolated from materials of gastric sites of diverse populations throughout the world (Owen, 1995). Over past two decades, increased attention had been paid on *H. pylori* since it plays important role in the pathogenesis of chronic gastritis and peptic ulcer diseases. Both epidemiological and clinical evidence has indicated that *H. pylori* is associated with an increased risk of gastric carcinoma (Heavey and Rowland, 2004). The International Agency for Research into Cancer (IARC) and the World Health Organization (WHO) had been classified this bacterium into Class I (definite) biological carcinogen in humans (Park *et al.*, 2004). Nowadays, *H. pylori* infection remains a worldwide spread disease with a definite morbidity and mortality (Zullo *et al.*, 2012). Moreover, the prevalence of infection in the developing countries was higher (greater than 80%) than the developed ones (less than 40%) (Vale and Vítor, 2010).

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European guidelines suggested the standard triple therapies including a proton pump inhibitor (PPI), clarithromycin (500 mg) plus amoxicillin (1 g) or

metronidazole/tinidazole (500 mg), all given twice daily for 7–14 days for *H. pylori* eradication (Zullo *et al.*, 2012). Unfortunately, decrease in the efficacy of this regimen was occurred throughout the world during the last decade. Consequently, the sequential therapy (10-days therapy) was proposed to get better results than triple therapies. This novel regimen consisted of a PPI plus amoxicillin 1 g (both twice daily) given for the first 5 days followed by a triple therapy including a PPI, clarithromycin 500 mg, and tinidazole (all drugs given twice daily) for the remaining 5 days. However, antibiotic-based treatment for *H. pylori* infection had higher effect on the composition of intestinal microbiota than probiotic-based treatment (Myllyluoma *et al.*, 2007). In addition, antibiotic-based therapy would not be cost effective, cause side effects and in particular, encouraging of widespread antibiotic resistance (Michetti, 2001). Currently, alternative or adjunctive therapy for the treatment of *H. pylori* infection was thus needed.

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There are several studies focusing on human- or dairy-originated probiotic lactic acid bacteria (LAB) since some strains of these bacteria exhibit antagonistic activity against *H. pylori*. Production of bacteriocins is one of the most important mechanisms that suppress growth of *H. pylori*. In addition to lactic acid and pH, bacteriocins with antagonistic activity against *H. pylori* might be produced by *Enterococcus faecium* TM39 (Tsai *et al.*, 2004). Heat-stable antimicrobial peptides secreted by *Bifidobacterium* strains could eradicate the growth of clinical strains of *H. pylori* and their effect were encouraged by organic acid produced during fermentative

metabolisms (Collado *et al.*, 2005). An *in vitro* study reported that nisin (class I), lacticin A164 (class II) and lacticin BH5 contained anti-*H. pylori* activity (Delves-Broughton *et al.*, 1996). Two bacteriocins including bulgaricin BB18 (produced by *Lb. bulgaricus* BB18) and enterocin MH3 (produced by *En. faecium* MH3) exhibited anti-*H. pylori* activity (Simova *et al.*, 2009). Pediocin BA28 (6.4 kB) secreted by *P. acidilactici* BA28 could suppress the growth of *H. pylori* (Kaur *et al.*, 2012). Hence, probiotics may represent a possible strategy for the treatment of *H. pylori* infection (Kaur and Garg, 2013).

Since plant-derived LAB are generally more resistant to severe conditions such as gastric juice and bile salts than animal-derived LAB. They can pass through the gastrointestinal tract and arrive to the intestine in the living state. Thus, they may be more functional as probiotics than animal-derived LAB (Higashikawa *et al.*, 2010). Therefore, the objectives of this study were focused on isolation and identification of plant-derived lactic acid bacteria, screening on their antibacterial activity, partial purification and characterization of antibacterial peptide and evaluation of probiotic property of the selected strains

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The main objectives of this research study are as followed:

1. To isolate, screen, and identify lactic acid bacteria with anti-*H. pylori* activity
2. To study the probiotic properties of selected strains
3. To purify and characterize the antibacterial peptide produced by selected strains

CHAPTER 2
LITERATURE REVIEW

2.1 Lactic acid bacteria (LAB)

Lactic acid bacteria are the group of Gram-positive, mostly catalase-negative, non-spore forming, devoid of cytochromes but aerotolerant, fastidious, acid-tolerant, cocci, coccobacilli or rod shape which produced lactic acid as the metabolic end product during fermentation of carbohydrates (Šušković *et al.*, 2010). They have less than 55 %mol G+C content in their DNA (Stiles and Holzapfel, 1997). They were normally found in various food products (milk, meat, beverages, and vegetables) and gastrointestinal tract of humans and animals. They comprised of several genera including *Lactobacillus* (*Lb.*), *Streptococcus* (*S.*), *Enterococcus* (*En.*), *Pediococcus* (*P.*), *Lactococcus* (*Lc.*), *Leuconostoc* (*Ln.*), *Aerococcus* (*A.*), *Oenococcus* (*O.*), *Carnobacterium* (*C.*), *Vagococcus* (*V.*), *Tetragenococcus* (*T.*) and *Weisella* (*W.*) (Axelsson, 2004). Among these genera, *Lactobacillus* is the largest genus (Parte, 2018). LAB can be divided into two groups based on their glucose metabolisms including homofermentative and heterofermentative LAB. Homofermentative LAB converted glucose almost quantitatively to lactic acid (more than 85%) through Embden-Meyerhof-Parnas or glycolysis pathway. Heterofermentative LAB fermented glucose to lactic acid (50%), ethanol/acetic acid, and CO_2 through phosphoketolase pathway (Reddy *et al.*, 2008).

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LAB have been found in various plant materials especially in plant surface and rotting vegetables and fruits. They play an important role for production of several fermented vegetables and fruits products such as silage, sauerkraut, kimchi, olive, pickles, beer and wine etc. Several species of LAB can be isolated from plants and fermented plant products including *Lb. plantarum*, *Lb. brevis*, *Lb. coryniformis*, *Lb. paracasei*, *Lb. curvatus*, *Lb. sakei*, *Lb. fermentum*, *En. faecalis*, *En. faecium*, *En. hirae*, *En. mundtii*, *En. casseliflavus*, and *En. sulfureus*, *Ln. mesenteroides* subsp. *mesenteroides* and subsp. *dextranicum*, *Ln. fallax*, *Ln. citreum*, *Ln. gelidum* and *Ln. kimchi* (Hammes and Hertel, 2009).

Economically, LAB play an important role in the production of fermented food such as fermented milk, meats, vegetables and cereal products. They are widely found as the natural microflora in both raw materials and fermented products and also play a major role in the digestive tract of humans and animals, especially in the young. (Stiles and Holzapfel, 1997). Due to lactic acid can use as a preservative and flavor additive in food industry as well as a precursor for the production of polylactic acid (PLA) used in the pharmaceutical and biodegradable plastic industries, some strains of LAB have been used as industrial producer of lactic acid (Gaspar *et al.*, 2013). Moreover, LAB are also used as "probiotics" since several studies revealed that LAB possess the beneficial effects on human health such as antimicrobial activity and gastrointestinal infections, effectiveness against diarrhea, improvement in lactose metabolism, antimutagenic properties, anticarcinogenic properties, reduction in serum cholesterol,

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Helicobacter pylori infection, inflammatory bowel disease and immune system stimulation (Shah, 2007).

2.2 LAB as probiotics

Probiotics is defined as "live microorganisms, which when administered in adequate amounts, confer a health benefit to the host" (FAO/WHO, 2002). The use of probiotics to improve intestinal health of humans has been purposed for many years (Servin and Coconnier, 2003). The heterogeneous group of LAB (*Lactobacillus*, *Enterococcus*, and etc.) and the genus of *Bifidobacterium* (*B.*) are known to commonly use as probiotics (Table 1) (FAO/WHO, 2002; Saad *et al.*, 2013). However, some genera including *Pediococcus* and *Leuconostoc*, have a limited number of probiotic properties (Shah, 2007).

Table 2.1 Lactic acid bacteria used as probiotics (Saad *et al.,* 2013*)*

Lactobacillus	***Bifidobacterium***	**Other LAB**
Lb. acidophilus	*B. adolescentis*	*E. faecium*
Lb. casei	*B. animalis*	*Lc. lactis*
Lb. crispatus	*B. bifidum*	*Ln. mesenteroides*
Lb. curvatus	*B. breve*	*P. acidilactici*
Lb. delbrueckii	*B. infantis*	*S. thermophilus*
Lb. farciminis	*B. lactis*	*S. diacetylactis*
Lb. fermentum	*B. longum*	*S. intermedius*
Lb. gasseri	*B. thermophilum*	
Lb. johnsonii		
Lb. paracasei		
Lb. plantarum		

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Selection of probiotic strains is based on the safety, probiotic (functional) properties and technological characteristics as presented in Figure 2.1 Human origin, non-pathogenicity and antibiotic resistance of strains are the important of safety consideration for selecting human probiotics (Saarela *et al.*, 2000). On the basis of probiotic properties, the ability to survive passage through the gut including resistance to gastric juices, exposure to bile and colonization on the digestive tract are the basic criteria for selection the probiotic strains (Saad *et al.*, 2013). Furthermore, immunostimulation (but no proinflammatory), production of antimicrobial substances, antagonistic activity against pathogenic bacteria (*Helicobacter pylori*, *Salmonella* sp., *Listeria monocytogenes* and *Clostridium difficile*), antimutagenic and anticarcinogenic properties are the other parameters assessed within under laboratory that effecting on human health (Saarela *et al.*, 2000).

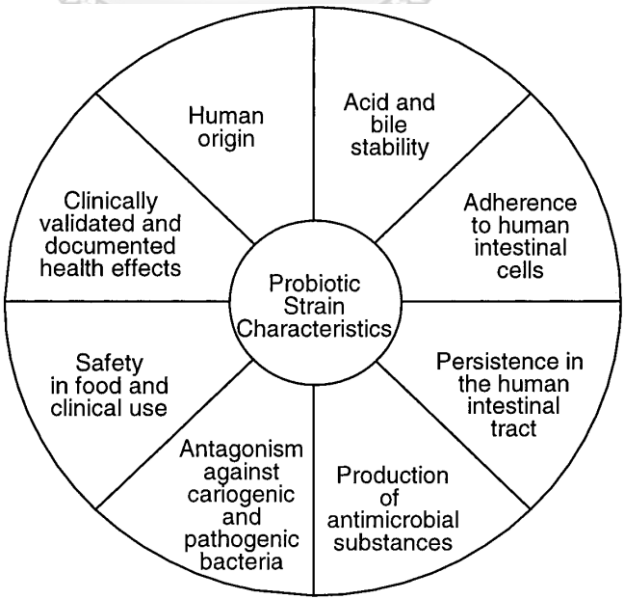

Figure 2.1 The theoretical basis for selection of probiotics includes safety, functional and technological aspects.

Although, almost microorganisms commercially used as probiotics are human or animal-derived LAB but screening for LAB strains from plant origin for potential probiotic features have become increased. Plant materials are occupied by lactic acid-producing microbiota which play a major role during fermentation of their fermented products. The important LAB in plant fermentation are belonged to the genus *Leuconostoc*, *Lactobacillus* and *Pediococcus* (Peres *et al.*, 2012). Recently, there was a study reported that some plant-derived LAB are more resistant than animal-derived LAB to simulated gastric juice and bile (Higashikawa *et al.*, 2010). Therefore, LAB appear to be probiotics for human consumption which have been extensively investigated (Peres *et al.*, 2012).

2.3 Probiotic properties of LAB

2.3.1 Antimicrobial activity

Among probiotic features, antimicrobial activity is one of the most important properties. Antimicrobial substances produced by LAB can be divided into 2 groups (Šušković *et al.*, 2010) including

1) Low molecular mass substances (<1,000 Da): organic acids, hydrogen peroxide, diacetyl, acetaldehyde and acetoin, carbon dioxide, reuterin, reutericyclin, cyclic dipeptides, phenyllactic acid, 4-hydroxyphenyllactic acid and 3-hydroxy fatty acids (Table 2.2)

2) High molecular mass substances (>1,000 Da): bacteriocins

Table 2. 2 Low molecular mass antimicrobial metabolites of lactic acid bacteria (Šušković *et al*., 2010)

Compound	Producers	Indicator strains
Lactic acid	All LAB	Yeasts
Acetic acid	Heterofermentative LAB	Yeasts
Diacetyl	Variety of genera of LAB including:	Yeasts
Hydrogen peroxide	All LAB	yeasts
Carbon dioxide	Heterofermentative LAB	Most of the taxonomic groups
Reuterin	*Lb. reuteri*	fungi, protozoa, Gram-positive
Reutericyclin	*Lb. reuteri*	Gram-positive bacteria
Cyclic dipeptides	*Lb. plantarum*	fungi
3-Phenyllactic acid	*Lb. plantarum*, *Lb. alimentarius*,	fungi
3-Hydroxy fatty acids	*Lb. plantarum*	fungi
Benzoic acid	*Lb. plantarum*	fungi

Some of LAB produce bacteriocins, antibacterial proteinaceous substances with bactericidal activity against related species (narrow spectrum) or across genera (broad spectrum of activity). Bacteriocin biosynthesis is a desirable characteristic for strain selection as it serves as an important mechanism of pathogen exclusion in fermented foods as well as in the gastrointestinal environment (Šušković *et al.*, 2010). *Lb. crispatus* and *W. koreensis* strains isolated from traditionally fermented south Indian koozh and gherkin (cucumber) exhibit a wide range of antimicrobial spectrum. The antimicrobial activity presented may be association with the production of antimicrobial peptides or bacteriocin-like substance of these strains. Moreover, broad-spectrum antimicrobial substance termed reuterin which first discover in 1988, is produced by *Lb. reuteri*

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resided in the gastrointestinal ecosystem of humans, poultry, swine, and other animals (Talarico *et al.*, 1988). This compound is capable to inhibit the growth of several pathogenic bacteria; *Escherichia*, *Salmonella*, *Shigella*, *Proteus*, *Pseudomonas*, *Clostridium* and *Staphylococcus* (Talarico *et al.*, 1988; Axelsson *et al.*, 1989). Some strains of *Lb. collinoides* can convert glycerol to reuterin during cider fermentation (Claisse and Lonvaud-Funel, 2000; Sauvageot *et al.*, 2000). Crude reuterin from *Lb. coryniformis* 394 isolated from silage can inhibit growth of yeast and mold involved in silage making (Nakanishi *et al.*, 2002).

2.3.2 Anti-*H. pylori* activity

An increased interesting of many researchers had been paid on LAB probiotics since some strains of these bacteria had exhibited antagonistic activity against *H. pylori*. Probiotic strains with this capability included *Lb. acidophilus*, *Lb. johnsonii* La1, *B. longum* BB536, *Lb. gasseri* OLL 2716, *Lb. salivarius*, *P. acidilactici* BA28 and etc. (Bazoli *et al.*, 1992; Michetti *et al.*, 1999; De Vrese and Schrezenmeir, 2002; Sakamoto *et al.*, 2001; Aiba *et al.*, 1998; Kaur *et al.*, 2012). Several mechanisms of *H. pylori* inhibition by probiotic bacteria had been proposed. Inhibition of adhesion *H. pylori*, maintaining of intestinal microbiota, decreasing inflammatory processes and production of non-immunological barriers such as short-chain fatty acids (SCFAs: acetic, propionic, butyric and lactic acids) and bacteriocins were the possible mechanisms of probiotics on inhibition of *H. pylori* (Anania *et al.*, 2016).

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However, little information involved anti-*H. pylori* activity of plant-derived LAB had been observed. LAB isolated from kimchi, a traditional Korean fermented vegetables, have been widely investigated for their anti-*H. pylori* activity. *Lb. plantarum* BK10, *Lb. brevis* BK11, *Lb. acidophilus* BK13, *P. pentosaceus* BK34, *Lb. paracasei* BK57, *E. faecalis* BK61 and *Lc. lactis* BK65 possess the significant antagonistic activity against *H. pylori* ATCC 43504. High concentration of lactic acid produced by *Lb. brevis* BK11, *Lb. acidophilus* BK13, *P. pentosaceus* BK34, *Lb. paracasei* BK57, and *Lb. lactis* BK65 reduce the viability of *H. pylori*. As a result of the ability to produce bacteriocin, *Lb. plantarum* BK10, *Lb. brevis* BK11, *Lb. acidophilus* BK13, *Lb. paracasei* BK57, and *E. faecalis* BK61 show the bactericidal effect against *H. pylori* ATCC 43504. Cell-free culture supernatant and bacteriocin solution collected from *E. faecalis* BK61 give the greatest effective for inhibiting the adhesion of *H. pylori* to human stomach adenocarcinoma cells when compare with other bacteriocin-producing LAB strains (Lim, 2014). In addition, *Lb. paraplantarum* KNUC25, a strain isolated from overfermented kimchi present the inhibition of *H. pylori* which is associated to acidic pH and reduction of adherence of *H. pylori* ATCC 43504 to gastric cells (Ki *et al.*, 2010). These researches indicated that plant-derived LAB can prevent the infection and colonization of *H. pylori* on the gastric mucosa and reduce the risk of gastrointestinal disease resulting from this pathogenic strain. Hence, plant-derived probiotics represent a possible strategy for the treatment of *H. pylori* infection.

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2.3.3 Adherence ability on gastric epithelial cell lines

H. pylori strain is able to tolerate very low acid conditions in the human stomach through the biosynthesis of urease and production of ammonium ions (Salama *et al.*, 2013). Besides, this bacterium has several adhesive factors that are important for protection against acidic pH, mucus and exfoliation during early and chronic phases of infection (Kalali *et al.*, 2014). Therefore, the adhesion is a desirable characteristic of interest for selection of probiotic strains because it is the first step in the prevention of colonization by the pathogens in the gastrointestinal tract due to competition for nutrients and adherence sites. For the past few years, several investigations reported that lactic acid bacteria and bifidobacteria had advantageous effects on the upper gastrointestinal tract. *S. thermophilus* CRL1190 strain had the ability to adhere to the stomach gastric mucosa, and improve protection against *H. pylori* through the reduction of its adhesion and the modulation of the inflammatory response (Marcial *et al.*, 2017). In addition, cells of probiotic *B. bifidum* YIT 10347 adhered to the human gastric mucosa in a live state, and that the higher adhering activity of *B. bifidum* YIT 10347 to the gastric mucosa may be involved in its beneficial effects on the human stomach (Shibahara-Sone *et al.*, 2016).

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2.3.4 Anticarcinogenic properties

Consumption of cured meat products, fats and low consumption of fiber make an important role on colorectal cancer in human. Many investigations also support that the colonic microflora are involved to cause carcinogenesis mediated by microbial

enzymes such as β-glucuronidase, azoreductase, and nitroreductase, which convert procarcinogens into carcinogens. *Lb. acidophilus* and *Bifidobacterium* spp. possess the ability to reduce the level of the enzymes responsible for converting procarcinogens to carcinogens, resulting in decrease the risk of tumor development (Shah, 2007). Moreover, LAB can reduce pro-carcinogen by assimilation nitrites and by reducing the levels of secondary bile salts (Fernandes and Shahani, 1990). Short-chain fatty acids (SCFA) especially, butyric acid produced by probiotics are nutrients and growth signal for intestinal epithelium and may responsible for colon cancer prevention. Rice bran-originated *Lb. plantarum* B2 can utilize rice bran as prebiotic substrate and convert it into SCFA (acetic, propionic and butyric acids). These organic acids can reduce the intestine pH, inhibit the pathogenic microorganism within intestine and prevent the colon cancer (Zubaidah *et al.*, 2012).

2.3.5 Reduction of serum cholesterol

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An important risk factor that impacts on cardiovascular disease (CVD) is high level of serum cholesterol particularly, low-density lipoprotein (LDL) (Chen and Konhilas, 2013). Several mechanisms involved the reduction of cholesterol by probiotics have been proposed. Deconjugation of bile acid by the action of bile salt hydrolase (BSH) from probiotics, bile acids are less soluble and absorbable by the intestines, leading to their elimination in feces. As a result, more cholesterol needs to be utilized to synthesize new bile acids, which in turn causes the lowering of serum cholesterol (Begley *et al.*, 2006). Cholesterol-binding ability of cellular membrane of

LAB cells has been proposed as another mechanism. Removing of cholesterol by growing cells is greater than dead cells, the heat-killed cells could still remove cholesterol from media, suggesting that some cholesterol was bound to the cellular surface (Usman, 1999). Five strains of *Lb. plantarum* (B282, E45, E10, E73, E79) and 7 strains of *Lb. pentosus* (B279, B283, B284, E43, E100, E106B, E129) isolated from fermented olive exhibit partial bile salt hydrolase activity (Argyri *et al.*, 2013). LAB isolated from traditionally fermented south Indian koozh and gherkin (cucumber) are used to test the bile acid deconjugating ability. The results found that *Lactobacillus* GI9, GI11 and *Weissella* FKI21 were able to deconjugate both sodium glycocholate and sodium taurocholate (Anandharaj *et al.*, 2015). Some plant-derived LAB have shown the ability to consume cholesterol *in vitro*. All strains of *P. pentosaceus* isolated from Idly batter exhibit the ability to assimilate cholesterol and especially, strain VJ13 has maximum cholesterol assimilation of 73% (Vidhyasagar and Jeevaratnam, 2013).

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2.4 Bacteriocins and their classification

Bacteriocins are ribosomally-synthesized peptides or proteins with anti-microbial activity that are produced by different groups of bacteria including many members of lactic acid bacteria (Gálvez *et al.*, 2007). Bacteriocins released from LAB are generally recognized as safe substances (GRAS); they have attracted interest to use extensively as food preservatives. Since nisin A was recognized in 1928, diverse bacteriocins have been identified from lactic acid bacteria (Zendo and Sonomoto,

2011). Interfering of cell wall and or the membrane of target organisms, either by inhibiting cell wall biosynthesis or causing pore formation, consequently resulting in death, are the proposed anti-microbial mechanism of bacteriocins (O'Sullivan *et al.*, 2002). Bacteriocins are classified into 4 class based on their structures and characteristics (Table 2.3)

2.5 Role of bacteriocins containing anti-*H. pylori* activity

LAB dominated in many fermented plant products could produce a diverse of different antimicrobial peptides that inhibit a wide range of Gram-positive and Gram-negative foodborne pathogens. Many bacteriocins from plant-derived LAB usually exhibit the antagonistic activity against Gram-positive bacteria such as *S. aureus*, *En. faecalis*, *L. monocytogenes*, *Bacillus coagulans* (Lim and Im, 2009; Lee *et al.*, 2007; Huang *et al.*, 2009; Masuda *et al.*, 2011). Only few of bacteriocins released from some plant-originated LAB (*En. faecium*, *Lc. Lactis*, *En. hirae* and *En. canis*) possess the inhibition against Gram-negative bacteria including *Escherichia coli* strains CI, CII, ATCC 25922 and ATCC 32158 (Ponce *et al.*, 2008). Although the studies on anti-microbial activity of bacteriocins produced from plant-origin LAB have become increased but however, effect of these substances on the growth of *H. pylori* and their characteristics are still less investigated. Purified bacteriocins produced from *Lb. brevis* BK11 and *En. faecalis* BK61 (isolated from Kimchi) show the significant inhibition on the growth of *H. pylori* ATCC 43504 (Lim, 2015). *Lb. bulgaricus* BB18 produced bacteriocin containing

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Table 2.3 Suggested classification of bacteriocins from lactic acid bacteria (Cotter *et al.,* 2005; Šušković *et al.,* 2010)

Classification	Major characteristics	Examples
Class I lantibiotics/lanthionine-containing bacteriocins subdivided into: Type A lantibiotics Type B lantibiotics	Small (<5 kDa) membrane-active peptides containing unusual amino acids; – elongated peptides with a net positive charge – smaller globular peptide with negative or no net charge	Type A : Nisin, Lacticin 481, Lactocin S Type B: Mersacidin
Class II non-lathionine-containing bacteriocins Subdivided into: Subclass IIa Subclass IIb Subclass IIc	Heterogeneous class of small (<10 kDa) heat-stable post-translation unmodified non-lantibiotics IIa: pediocin-like IIb: two-peptide IIc: with wide range of effects on membrane permeability and cell wall formation	IIa: Pediocin PA1, Sakacin A, Sakacin P, Leucocin A, Curvacin A IIb: Lactococcin G, Lactococcin M, Lactacin F, Plantaricin A IIc: Acidocin B, Enterocin P, Enterocin B, Reuterin 6
Class III Bacteriolysins	Large (>30 kDa) heat-labile antimicrobial proteins complex proteins with domain-type structure that function through the lyses of sensitive cell by catalyzing cell wall hydrolysis	Lysostaphin, Enterolysin A, Helveticin J, Helveticin V-1829
Class IV	Complex bacteriocins carrying lipid or carbohydrate moieties	Plantaricin S, Leuconocin S, Lactocin 27, Pediocin SJ1

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anti-*H. pylori* activity (Simova *et al.*, 2009). Bacteriocins including Nisin A, Lacticin A164, Lacticin BH5, Lacticin JW3, Lacticin NK24, Pediocin PO2 and Leucocin K exhibit the different level of suppression on *H. pylori* growth (Kim *et al.*, 2003). At present, bacteriocins are widely used as food preservatives for extending shelf-life of food, bacteriocin-producing probiotics can balance the microflora in the gastrointestinal tract to reduce gastrointestinal diseases. Due to the antimicrobial activity against *H. pylori*

strains of several bacteriocins, they can be developed as an attractive substitute to one or more antibiotics in clinical treatments and would have potential application in treating stomach ulcers (Kim *et al.*, 2003). Moreover, bacteriocins have the potential to use in pharmaceutical industry for replacing or reducing the use of antibiotics (Yang *et al.*, 2014).

2.6 Characteristics of bacteriocins

Some characteristics including molecular weight, amino acid sequence, antimicrobial spectrum, and some factors affecting anti-microbial activity of peptides are usually investigated.

2.6.1 Molecular weight

The molecular mass and amino acid sequencing are used to identify or classify the bacteriocins. The molecular mass of normal purified bacteriocins ranged from 2500 Da to 6500 Da (Elegado *et al.*, 1997; Ennahar *et al.*, 2000). After purification, two purified bacteriocins with anti-*H. pylori* activity from *Lb. brevis* BK11 and *E. faecalis* BK61 were estimated to be about 6.5 and 4.5 kDa, respectively (Lim, 2015). Molecular weight of pediocin BA28 produced from *P. acidilactici* BA28 that contains anti-*H. pylori* activity, is about 6.4 kDa (Kaur and Garg, 2013). The approximate molecular mass of bulgaricin BB18 with anti-*H. pylori* activity from *Lb. bulgaricus* BB18 is about 4.2 kDa. Not only its molecular weight, but also its N-terminal sequencing always be used to identify or classify the type of bacteriocins. Bulgaricin BB18 secreted by *Lb. bulgaricus*

BB18 contains 31 amino acid residues in N- terminal in the order of KIYRGNVGHCGKSTVDWGTAIGNGNNAASFL and it was classified to class IIa nonlantibiotics bacteriocins (Simova *et al.*, 2009).

2.6.2 Antimicrobial spectrum of bacteriocins

Bacteriocins produced by LAB generally possess antibacterial activity against Gram- positive foodborne pathogens such as *Bacillus* spp. , *Clostridium* spp. , *Staphylococcus* spp. and *Listeria* spp. (Zendo and Sonomoto, 2011). Many strains of plant-derived LAB could produce bacteriocins with antimicrobial activity against Gram-positive bacteria. Paraplantaricin C7 produced from *Lb. paraplantarum* C7 (isolated from Kimchi) exhibit antibacterial activity against gram- positive bacteria such as *Ln. mesenteroides*, *Lb. delbrueckii* subsp. *lactis*, *Lb. pentosus*, *Lb. plantarum* and *E. feacalis* (Lee *et al.*, 2007). *P. pentosaceus* 05-10, isolated from a traditionally fermented Sichuan Pickle, produced a bacteriocin (Pediocin 05-10) with antagonistic activity against *Listeria*, *Lactobacillus*, *Streptococcus*, *Enterococcus*, *Pediococcus* and *Leuconostoc* (Huang *et al.*, 2009). Lactocyclicin Q obtained from *Ln. mesenteroides* TK41401 (isolated from Japanese pickle) display the antimicrobial activity against *Lactococcus*, *Lb. sakei*, *W. paramesenteroides*, *P. dextrinicus*, *Enterococcus*, *Streptococcus*, *Leuconostoc* and *B. coagulans* (Masuda *et al.*, 2011).

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Recently, broad spectrum antimicrobial activities of plant- derived LAB bacteriocins against foodborne pathogens have been reported. Bacteriocins produced by LAB isolated from organic vegetables are active against *L. monocytogenes* and *E.*

coli (Ponce *et al.*, 2008). Purified bacteriocins obtained from *Lb. brevis* BK11 exhibit broad spectrum antimicrobial activity against *Lb. acidophilus*, *Lb. brevis*, *Lb. plantarum*, *H. pylori* and *S. enteritidis*. Purified bacteriocin produced by *En. faecalis* BK61 show the antimicrobial against *Lb. acidophilus*, *Lb. brevis*, *Lb. paracasei*, *En. faecalis*, *L. monocytogenes*, *S. aureus*, *E. coli* O157: H7, *H. pylori* and *S. enteritidis* (Lim, 2015).

2.6.3 Stability of bacteriocin containing anti-*H. pylori* activity

2.6.3.1 Temperature

The effect of various temperatures on anti-*H. pylori* activity of bacteriocins has been studied. After heat treatment of bacteriocin produced by *Lb. brevis* BK11 at 100 °C for 10, 20 and 30 min, the antimicrobial activity was not altered. However, heating at 121 °C for 15 min results in reduction of anti-*H. pylori* activity up to 25%. Bacteriocins produced by *En. faecalis* BK61 exhibit good stability to heating at 100 °C for 10 and 20 min, but the activity was partially decreased when exposes to heating at 100 °C for 30 min and 121 °C for 15 min (Lim, 2015). Anti-*H. pylori* activity of bacteriocin produced by *Lb. bulgaricus* BB18 show the stability to heating at 100 °C and 115 °C for 60 min. After heat treatment at 121 °C for 15 min, the activity of bacteriocin is slightly decreased (Simova *et al.*, 2009). In addition, crude bacteriocin obtained from *E. faecium* TM39 is resistant to heating at 100 °C for 15 min (Tsai *et al.*, 2004). The effect of heat treatment (80 °C for 15, 20 and 60 min and 100 °C for 10 and 20 min) on anti-*H. pylori* activity of crude bacteriocin produced by *Bifidobacterium*

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strain was studied. The results found that bacteriocin possess the stability to heating at 100 °C for 10 min (Collado *et al.*, 2005).

2.6.3.2 pH

A few studies have investigated the effect of pH on anti-*H. pylori* activity of bacteriocins. Purified bacteriocin secreted from *Lb. brevis* BK11 exhibited the good stability to a wide range of pH from 2 to 12. *En. faecalis* BK61 isolated from Kimchi, produced bacteriocin that had the stability over a pH range from 4 to 10 (Lim, 2015). Bacteriocin produced by *Lb. bulgaricus* BB18 displayed the inhibitory activity within a wide range of pH from 3 to 11 (Simova *et al.*, 2009). Crude bacteriocin obtained from *Bifidobacterium* strain was stable over a wide range of pH from 3-10 (Collado *et al.*, 2005).

2.6.3.3 Enzymes

Many enzymes including lipases, amylase, and proteases that usually presented in foods were used to evaluate the effect of these enzymes on anti-*H. pylori* activity of AMPs. Anti-*H. pylori* activity of AMP produced from *Lb. brevis* BK11 was significant decreased when this substance was exposed to protease, proteinase K, trypsin, and lysozyme. Pepsin, α-amylase and lipase were not affect on anti-*H. pylori* activity of this substance. Bacteriocin produced by *En. faecalis* BK61 was completely inactivated by protease and loss its activity was observed after treatment with protease K, trypsin and pepsin but it was insensitive to lysozyme, α-amylase and lipase (Lim, 2015). Anti-*H. pylori* activity of bacteriocin obtained from *Lb. bulgaricus* BB18 was

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inactivated by proteinase K, protease IX and XIV, trypsin, α-chymotripsin and pepsin but it was insensitive to α-amylase and lipase (Simova *et al.*, 2009). Crude bacteriocin does not loss its anti-*H. pylori* activity after treated with pepsin, trypsin, chymotrypsin and proteinase K (Tsai *et al.*, 2004). The inhibitory activity of crude bacteriocin from *Bifidobacterium* strain was inactivated by trypsin, protease A and pepsin (Collado *et al.*, 2005).

2.6.3.4 Storage

Evaluation of storage conditions on the stability of bacteriocins have studied. The anti-*H. pylori* activity of bacteriocins produced from *Lb. brevis* BK11 and *En. faecalis* BK61 were stable during storage time for 3 months at 25 and 4 ℃. The inhibitory activity of these bacteriocins exhibited the complete stability at -20 ℃ for 60 days of storage period. However, they were approximately 50% loss of activities of these bacteriocins after 90 days of storage at the same temperature (Lim, 2015). bacteriocin obtained from *Lb. bulgaricus* BB18 retained the maximum activity during 90 days of storage at -20 ℃ (Simova *et al.*, 2009).

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CHAPTER 3

RESEARCH METHODOLOGY

3.1 Media, Chemicals and Equipments

1) Lactobacilli MRS broth, Difco, USA

2) Columbia blood agar

3) Skimmed milk, Difco, USA

4) RPMI 1640 medium, Gibco, USA

5) Fetal bovine serum, Corning™, USA

6) Fetal horse serum, Gibco, USA

7) Sheep blood, Faculty of Veterinary Science, Chulalongkorn University, Thailand

8) Calcium carbonate ($CaCO_3$), Merck, Germany

9) AnaeroPack™-MicroAerobic and Anaerobic gas generator kit, Mitsubishi, Japan

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10) Bovine serum albumin (BSA), Sigma-Aldrich, USA

11) Trizma® base, Sigma-Aldrich, USA

12) 37% Hydrochloric acid (HCl), Merck, Germany

13) Sodium chloride (NaCl), Sigma-Aldrich, USA

14) Sodium hydroxide, Merck, Germany

15) Methanol, Merck, Germany

16) Pyridine, Merck, Germany

17) α-Cyano-4-hydroxycinnamic acid, Sigma-Aldrich, USA

18) Trifluoroacetic acid (TFA), Sigma-Aldrich, USA

19) Iso-propanol, Merck, Germany

20) Acetonitrile HPLC grade, Fisher Chemical, USA

21) Oxgall, Sigma-Aldrich, USA

22) API 50 CH, bioMérieux, USA

23) Amberlite^{TM} XAD-16 resin, Sigma-Aldrich, St Louis, MO, USA

24) SP-Sepharose Fast Flow cation-exchange column, GE Healthcare, Uppsala, Sweden

25) Universal primers for 16S rRNA gene sequence:

- 27F (5'-AGAGTTTGATCMTGGCTCAG-3'),
- 337F (5′-GACTCCTACGGGAGGCWGCAG-3′)
- 518F (5′CCAGCAGCCGCGGTAATACG-3′)
- 785F (5′-GGATTAGATACCCTGGTA-3′)
- 800R (5′-TACCAGGGTATCTAATCC-3′)
- 907R (5′-CCGTCAATTCMTTTRAGTTT-3′)
- 1100R (5′-GGGTTGCGCTCGTTG-3′)
- 1492R (5'-TACGGYTACCTTGTTACGACTT-3')

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26) Primers for phenylalanyl-tRNA synthase (*pheS*) gene:

- *pheS-21-F* (5'-CAYCCNGCHCGYGAYATGC-3')
- *pheS-23-R* (5'-GGRTGRACCATVCCNGCHCC-3')

27) *Taq* DNA polymerase and buffer system, Vivantis Technologies, Malaysia

28) 10-kb DNA molecular weight marker, Vivantis Technologies, Malaysia

29) 24-well plate, NUNC, Thermo Scientific, USA

30) Sterile 0.2 μm hydrophilic PTFE syringe filters, 13 mm, Advantec

31) Thin-layer chromatography (TLC) plate, Millipore, USA

32) Incubator, Model: BE600, Memmert, Germany

33) Autoclave, Model: HA-3D, Hirayama, Japan

34) PCR Authorized Thermal Cycler, C1000 Touch™, Bio-Rad Laboratories Inc., Berkeley, California

35) Mini-Run Gel Electrophoresis System, Model: GE-100, Hangzhou Bioer technology, China

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36) Gel Documentation system, Bio-Rad Laboratories Inc., Berkeley, California

37) Laminar flow hood, Model: BV-126, ISSCO, Thailand

38) Vortex mixer, Model: Vortex-Genie 2, Scientific Industries, USA

39) pH meter, Model: SevenEasy™, METTLER TOLEDO, Italy

40) Electronic analytical balance, Model: AB204-S, METTLER TOLEDO, Italy

41) Stirrer, Model: IKA® C-MAG HS-7

42) Thermo mixer C, Eppendorf, Germany

43) Centrifuge 5810 R, Eppendorf, Germany

44) Centrifuge, Model: Avanti J-E, Beckman Coulter

45) Certomat BS-1 Refrigerated Incubator Shaker, Sartorius, Germany

46) Resource RPC column, GE Healthcare, Sweden

47) LC-2000 Plus High Performance Liquid chromatography system (Jasco, Tokyo, Japan)

48) Freezing container, Nalgene® Mr. Frosty, Thermo Scientific, USA

49) Phase contrast microscopy (ZEISS Primo Star, USA)

50) Inverted microscope, Nikon model TMS, Japan

51) CO_2 incubator, NAPCO 6000, Thermo Scientific, USA

52) Microaerobic chamber, Sheldon Manufacturing Inc., Cornelius, Oregon, USA

3.2 Methodology

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3.2.1 Experimental plan

LAB strains were isolated from plant and fermented food products collected in Thailand. All strains were screened for their anti-*H. pylori* activity. The strains containing anti-*H. pylori* activity were identified. Adherence ability of selected strains were determined. The nature of anti-*H. pylori* compound produced from selected strains were evaluated. Partial purification of bacteriocins produced from a selected strain was carried out.

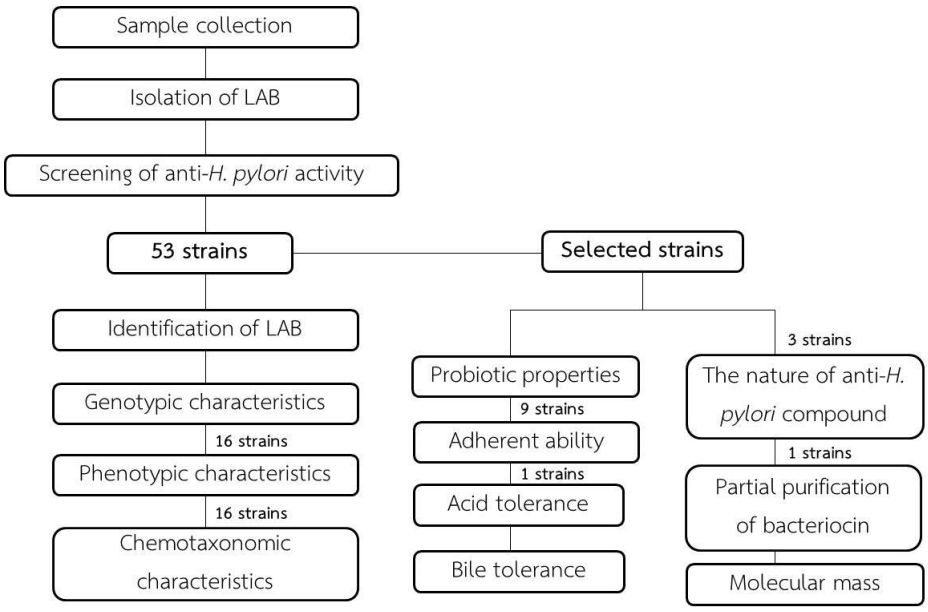

Figure 3.1 Experimental flow chart of this study

3.2.2 Sample collection and bacterial isolation

Generally, LAB isolated from plant materials were more resistant to severe conditions such as gastric fluids and bile acids in the gastrointestinal tract of human than those isolated from animal sources (Higashikawa *et al.*, 2010). In addition, plant materials composed of sugars which could be utilized by LAB. Therefore, plant and fermented food products; West-Indian jasmine, coffee cherries, mushroom, fermented tea leaves (Miang), fermented plant beverages, fermented mushroom and fermented rice noodle (Khanom-jeen) which were collected in Thailand, were used as samples for LAB isolation in this study. 0.5 g of each sample was aseptically transferred to 5 mL of De Man Rogosa and Sharpe (MRS) broth (De Man *et al.*, 1960) and incubated at 30 °C for 48 h under aerobic condition. For purification step, one loopful of culture broth was streaked on MRS agar containing with 0.3% calcium carbonate ($CaCO_3$) and

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incubated under the same conditions. A single colony surrounding with clear zone was picked up and cultivated in MRS broth. This procedure was repeated until obtained the pure cultures. Pure cultures were maintained at -80 °C in 10% skim milk and lyophilized for further analysis.

3.2.3 Screening of antibacterial activity

3.2.3.1 Cultures and conditions

Stock cultures of LAB were cultivated on MRS agar and incubated at 30 ºC for 24 h. A single colony grown on MRS agar was propagated in MRS broth at 30 ºC for 24 h. *H. pylori* strains 2649, MS83, and BK364 isolated from a patient suffering from gastritis, peptic ulcer diseases, and gastric cancer, respectively were used as the indicator strains. These three clinical *H. pylori* strains were obtained from Faculty of Medicine, Thammasat University, Pathum Thani, Thailand. *H. pylori* strains were cultivated on Columbia blood agar supplemented with 7% sheep blood and incubated at 37 ºC under microaerobic conditions using a gas generating kit (Anaero Pack-MicroAero, Mitsubishi Gas Chemical, Japan) for 72 h. They were subcultured twice on the same medium before experimental use.

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3.2.3.2 Determination of antibacterial activity

All LAB strains were cultivated at 30 ºC for 24 h in MRS broth and cells were removed by centrifugation at 14,000 rpm for 10 min at 4 ºC. The supernatants were collected, adjusted pH to 6.5 with 3 M Tris and then, filtered through 0.22 µm pore-size sterile filter. The anti-*H. pylori* activity was carried out by the spot-on-lawn method.

This assay was performed by spotting 10 μl of cell-free supernatant (CFS) onto a double layer including 5 ml of brain heart infusion (BHI) agar (0.7% agar) containing 10^7 cfu/ml of *H. pylori* culture which overlaid on the BHI agar medium (1.5% agar). After incubation under microaerobic conditions at 37 ºC for 72 h, the inhibition zone (mm) on *H. pylori* lawn was measured (Ennahar *et al.*, 2001).

3.2.4 Identification of LAB

3.2.4.1 Phenotypic characteristics

After growing on MRS agar plates at 30 ºC for 48 h, the morphological characteristics were determined (Tanasupawat *et al.*, 2007) as followed;

- Cell shape
- Cell size
- Cell arrangement
- Colonial appearance

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Cells were cultivated in MRS broth at 30 ºC for 48 h. The biochemical characteristics were evaluated as followed (Appendix B);

- Gram staining (Hucker and Conn, 1923)
- Catalase test
- Nitrate reduction
- Gas production
- L-Arginine hydrolysis
- Starch hydrolysis

- Slime formation
- Diacetyl production (Phalip *et al.*, 1994)
- Acid production from carbohydrates by using API 50 CH (bioMérieux)
- Lactic acid isomer (Okada *et al.*, 1978)

Physiological characteristics were also determined as followed;

- Growth at different pH (3.0–9.0, using relevant buffers)
- Growth at different temperatures (20, 25, 30, 37,40, 42, 45 and 50 ºC)
- Growth in different NaCl concentrations (1, 2, 3, 5, 7 and 9 %, w/v)

3.2.4.2 Chemotaxonomic characteristics

3.2.4.2.1 Cell wall composition

Cells were grown in MRS broth at 30 ºC for 48 h, harvested by centrifugation at 10,000 rpm for 15 min and then washed twice with 0.85% NaCl solution. The amino acids in the cell wall was detected by TLC technique (Hasegawa *et al.*, 1983). Briefly, cells pellet was hydrolyzed by using 0.1 ml of 6N HCl (hydrochloric acid) and heated by autoclaving at 121 ºC for 15 min. After cooling, 1 μl of hydrolysate was spotted onto a cellulose TLC plate. The TLC plate was developed with the solvent system of methanol-water-6N HCl-pyridine (80: 26: 4: 10 v/v) for 3 h. After the second developing, the TLC plate was sprayed with Ninhydrin spray reagent and heated at 100 ºC for 2 min to visualize the spot. *Meso*- diaminopimelic acid (DAP), *LL*- DAP L- lysine

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hydrochloride, D-aspartic acid, L-glycine, L-alanine and DL-serine was used as the standard.

3.2.4.2.2 Cellular fatty acid analysis

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

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3.2.4.2.3 DNA base composition analysis

DNA was isolated and purified from cells grown in MRS broth supplemented with 0.3% glycine after incubating at 30 ºC for 24 h (Yamada and Komagata, 1970; Tamaoka, 1994). Cells pellet was suspended in 2 ml of saline-EDTA with 5-10 mg of

lysozyme and incubated at 37 ºC for 30 min. The mixture was added with 0.1 M Tris and 0.1 M NaCl, pH 9 (8 ml) and 10% sodium dodecyl sulphate (SDS) (0.05-0.1 ml) and then heated at 55-60 ºC for 10 min. In order to separate the proteins, phenol-chloroform (1:1) solution (5 ml) was added to the mixture, shaked for several minutes 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 0.1X SSC (0.1 M NaCl and 0.015 M Na-Citrate, pH 7) (3 ml). For RNA elimination, the sample was added with RNase A solution (0.3 ml) and incubated at 37 ºC for 20 min. The DNA sample was added with 10X SSC solution (0.5 ml) and extracted again 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 0.1X SSC. 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 DNA sample (10 μg/ml) was heated at 100 ºC for 10 min. The denatured DNA was added with 10 μl of nuclease P1 solution and incubated at 50 ºC for 1 hour and then, added 10 μl of alkaline phosphatase solution, incubated at 37 ºC for 1 hour. DNA base composition was determined by using reversed-phase HPLC (Tamaoka and Komagata, 1984).

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3.2.4.3 Genotypic characteristics

The 16S rRNA gene was amplified by polymerase chain reaction (PCR) using the primers 20F (5′-AGTTTGATCCTGGCTC-3′) and 1530R (5′-AAGGAGGTGATCCAGCC-3′). Amplification reaction was carried out in 50 μl volume, using *Taq* DNA polymerase and buffer system (Vivantis Technologies, Malaysia). The final PCR mixture consisted of 10× ViBuffer A (without $MgCl_2$; 50 mM KCl, 50 mM Tris-HCl (pH 9.1 at 20 ºC) and 0.1% Triton™ X-100), 5 mM $MgCl_2$, 0.2 μM concentration of each deoxynucleoside triphosphate, 0.2 μM concentration of each primer, one unit of *Taq* DNA polymerase, and 50 ng of template DNA. Amplification was carried out in a thermocycler (C1000 Touch™, Bio-Rad Laboratories Inc., Berkeley, California) with the following cycling program: initial denaturation at 94 ºC for 5 min, followed by 40 cycles of 94 ºC for 2 min, annealing at 55 ºC for 1 min, and extension at 72 ºC for 2 min, and a final extension step at 72 ºC for 5 min (Kawasaki *et al.*, 1993). Three microliters of the PCR product was electrophoresed for 40 min at 100 Volts on a 1.5% (w/v) agarose gel in 1× TAE buffer (40 mM Tris base (pH 7.6), 20 mM acetic acid, 1 mM EDTA, pH 8.0). A 10-kb DNA molecular weight marker (Vivantis Technologies, Malaysia) was included as standard for the calculation of the fragments. After staining with ethidium bromide (0.5 μg ml^{-1}) for 5 min, the fingerprint patterns were visualized under ultraviolet light followed by digital capture using the Gel Documentation system (Bio-Rad Laboratories Inc., Berkeley, California). PCR products were sequenced (Macrogen, Seoul, Korea) by using the universal primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′), 337F (5′-

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GACTCCTACGGGAGGCWGCAG-3′), 518F (5′CCAGCAGCCGCGGTAATACG-3′), 785F (5′-GGATTAGATACCCTGGTA-3′), 800R (5′-TACCAGGGTATCTAATCC-3′), 907R (5′-CCGTCAATTCMTTTRAGTTT-3′), 1100R (5′-GGGTTGCGCTCGTTG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′) (Lane, 1991). The 16S rRNA gene sequences were multiple-aligned with the selected sequences obtained from the EzTaxon-e database (Yoon *et al.*, 2017) by using the CLUSTAL_X program (Thompson *et al.*, 1997). Gaps and ambiguous bases were eliminated before reconstructing the phylogenetic tree by the neighbor-joining method (Saitou and Nei, 1987) in the MEGA 6 program (Tamura *et al.*, 2013). Confidence values of the branches of phylogenetic tree were evaluated by using the bootstrap resampling method with 1000 replications (Felsenstein, 1985).

3.2.5 Probiotic properties

3.2.5.1 Adherence assay

3.2.5.1.1 AGS cell line and culture conditions

Human gastric adenocarcinoma epithelial cell line AGS (ATCC® CRL 1739™) was purchased from American Type Culture Collection (ATCC). Cells were routinely cultivated in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco™, Thermo Scientific, USA) supplemented with 10% FBS in an incubator (NAPCO 6000, Thermo Scientific, USA) with 95% humidified air and 5% CO_2 (v/v) at 37 °C for 4-5 days. For passaging, cells monolayers were detached for 10 min using 0.25 M trypsin-EDTA (Gibco™, Thermo Scientific, USA) and then washed with sterile phosphate buffered saline (PBS, pH 7.4). Cells were seeded at 1×10^5 cells/well in 24-well tissue culture

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plates (Nunc, Thermo Scientific, USA) with or without placing 12-mm diameter glass coverslips and added 0.5 ml of RPMI medium supplemented with 10% FBS. Then, the plates were incubated at 37 ºC with 5% CO_2/95% humidified air until cells reached confluency (4-5 days).

3.2.5.1.2 Determination of adherence ability of LAB

After AGS monolayers cultured in 24-well plates were confluent, they were washed once with PBS to remove the unattached cells. Cells of selected LAB strains were prepared in the RPMI supplemented with 10% FBS to achieve the cell concentration of 10^8 cfu/ml was achieved. 0.5 milliliter of each bacterial suspension was added to each wells of a tissue culture plate and then incubated at 37 ºC for 90 min under 5% CO_2 atmosphere. After incubation, the monolayers were washed 3 times with PBS to remove non-adhered bacterial cells. Bacterial cells were counted by cultural and microscopic enumeration.

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3.2.5.1.3 Cultural enumeration

AGS cells were detached by addition of 0.5 ml of triton X-100 and left at room temperature for 30 min. The cell suspensions of LAB were serially diluted and counted by spread plate method after incubation at 37 ºC for 48 h. The experiments were performed in triplicate. Adherent rate of selected LAB strain (%) was calculated as followed;

Adherent rate (%) = (The number of bacteria adhered/the number of bacteria added) ×100

3.2.5.1.4 Microscopic enumeration

After fixing the coverslips with 100% methanol for 20 min, adhered cells were then stained with crystal violet solution for 1 min. The coverslips were washed once with distilled water and dried at room temperature. The adhered cells onto coverslips were observed under a phase contrast microscope (ZEISS Primo Star, USA) with 1000x magnification. Adherent rates of LAB were expressed as adhered bacteria per cells.

3.2.5.2 Resistance to low pH

Acid tolerance was determined as a minor modification of the previous report (Makete *et al.*, 2017). Single colony of selected LAB strain was anaerobically cultivated in MRS broth at 37 ºC for 18 h. Cells (4.0×10^9 cfu/ml) were centrifuged (8,000 × *g* for 5 min at 4 ºC), washed twice with sterile normal saline (0.85% NaCl), and re-suspended in 10 ml of MRS broth. Cell suspension (1%) was inoculated into 10 ml of MRS with adjusting pH to 2.0 and 3.0 using 6 N hydrochloric acid (HCl). The cultures were then anaerobically incubated at 37 ºC. At 0, 1.5, and 3 h of incubation, viable cell counts were determined on a MRS agar plate using the spread plate technique.

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3.2.5.3 Resistance to bile salts

Bile tolerance was evaluated with some modifications (Thamacharoensuk *et al.*, 2013). One hundred microliters of cell suspension were inoculated into 10 ml of MRS broth supplemented with 0.3% and 0.5% of oxgall (Sigma-Aldrich, MD, USA). The cultures were then anaerobically incubated at 37 ºC. After incubation at 0, 3, and

6 h, viable cell counts were examined on a MRS agar plate using the spread plate technique.

3.2.6 Characterization of antimicrobial compound

LAB strains which showed the high anti-*H. pylori* activity was selected to determine the nature of compounds responsible for antimicrobial activity. To exclude the effect of organic acids, CFS collected from the selected strain was neutralized to pH 6.5 by using 3 M Tris base. In addition, CFS was treated with different enzymes including pepsin, trypsin, α- chymotrypsin, proteinase K, and catalase at final concentration of 1 mg/ml. After incubation for 3 h under optimal pH and temperature for each specific enzyme, the anti-*H. pylori* activity of enzyme-treated samples was tested by critical dilution spot-on-lawn assay. The anti-*H. pylori* activity was expressed as arbitrary activity units (AU) per milliliters which were defined as the reciprocal of the highest dilution causing a clear zone of inhibition in the indicator lawn according to the following formula;

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$$\text{The anti-}H.\ pylori\ \text{activity (AU/ml)} = 2^{N} \times 100$$

where, AU is the arbitrary unit and N is the highest two-fold serial dilution showing an inhibition zone of the bacterial indicator strain.

3.2.7 Partial purification of bacteriocin from the selected strain

3.2.7.1 Purification procedure

Bacteriocin purification was performed by Woraprayote. Briefly, a selected producer strain, *Ln. pseudomesenteroides* FM-S16, was cultivated in MRS broth (1 L)

at 30 ºC for 18 h. The cell-free culture supernatant was harvested by centrifugation at 8,000 x *g* for 15 min at 4 ºC. Amberlite™ XAD-16 resin (25 g) (Sigma-Aldrich, St Louis, MO, USA) prevoiusly activated with 50% (v/v) isopropanol was mixed with the cell-free culture supernatant and gently shaken at 4 ºC for 24 h. Then, the mixture was subjected to a column (25 mm internal diameter, 300 mm length) and serially washed with 200 ml of MilliQ water and 200 ml of 50% ethanol. Bacteriocin was eluted with 100 ml of 70% isopropanol containing 0.1% trifluoroacetic acid (TFA). The isopropanol in the active fraction was removed by evaporation with a rotary evaporator. The resulting solution was diluted with 20 mM sodium phosphate buffer (pH 5.7) to 100 ml and subjected onto a SP-Sepharose Fast Flow cation-exchange column (15 mm internal diameter, 100 mm length; GE Healthcare, Uppsala, Sweden) pre-equilibrated with 20 mM sodium phosphate buffer (pH 5.7). The column was serially washed with 100 ml of the 20 mM sodium phosphate buffer, 20 mM sodium phosphate buffer containing 0.25, 0.5, 0.75 and 1 M NaCl (pH 5.7). The active fraction was loaded onto a reverse-phase column (3-ml RESOURCE RPC column; Amersham biosciences, Uppsala, Sweden) equipped with high performance liquid chromatography and eluted with a gradient of MilliQ water-acetonitrile containing 0.1% TFA at a flow rate of 1 ml/min as follows: 0-0.1 min, 5-30% acetonitrile; 0.1-10 min, 30% acetonitrile; 10-35 min, 30-80% acetonitrile; 35-40 min, 80-100% acetonitrile and 40-50 min, 100% acetonitrile. Absorbance was monitored at 220 nm (Woraprayote *et al.*, 2015). All peaks

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of interest were collected and identified for the anti-*H. pylori* activity by spot-on-lawn technique as previously described.

3.2.7.2 Protein determination

Protein concentration was determined by Pierce™ BCA protein assay kit (Thermo Scientific™, USA) using bovine serum albumin (BSA) as standard.

3.2.7.3 Molecular weight

The molecular mass of antibacterial peptide was determined by the matrix-assisted laser desorption/ionization time of flight (MALDI-TOF/TOF) mass spectrometry in Ultra-flex III TOF/TOF spectrometer equipped with laser (337 nm) in reflector mode and calibrated using ACTH fragment 18-39 (human), insulin oxidized B chain (bovine), and insulin (bovine) standards. A 1 µl aliquot of the mixture was mixed with 1 µl CHCA solutions (10 mg of α-cyano-4-hydroxycinnamic acid in 1 ml 0.1% TFA in acetonitrile). A 2 µl aliquot of the resulting mixture was applied on the stainless steel MALDI target and dried in air. At least 1000 laser shots were collected for each spectrum and the data was analyzed using the Flax Analysis 3.0 software

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CHAPTER 4
RESULTS AND DISCUSSION

4.1 Isolation of LAB

Table 4.1 showed a total of 238 bacteria which were isolated from various plant-based products including West-Indian jasmine from Kalasin (1 strain), coffee cherries from Chiang Mai (9 strains), mushroom from Phrae (11 strains), Lamphun (23 strains) and Lampang (71 strains), fermented tea leaves (*Miang*) from Chiang Mai (7 strains) and Lampang (12 strains), fermented plant beverage from Chiang Mai (28 strains), fermented mushroom from Nakhonratchasima (12 strains), and fermented rice noodle (*Khanom-jeen*) from Songkhla (5 strains) and Nakhonratchasima (27 strains) by using MRS medium. The results revealed that plant materials and fermented food products were the good sources for LAB isolation. All LAB strains were Gram-positive, catalase-negative, non-motile and non-spore forming bacteria. Generally, LAB could be found in rich nutrient environments such as food products (milk, meat, beverages, vegetables), but some are also members of the normal flora of the mouth, intestine, and vagina of mammals (Axelsson, 2004). Due to fruits and vegetables are rich in sugar, water-soluble vitamins, phytosterols, phytochemicals, dietary fibers and minerals and have suitable pH, and thus, provide a natural medium for lactic acid fermentation by LAB (Swain *et al.*, 2014).

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Table 4.1 Sample, Location, strain number and number of strain of LAB

Sample	Location	Strain no.	No. of strain
West-Indian jasmine[a]	Kalasin	Ru20-1	1
Coffee cherries[b]	Chiang Mai	CCA-1, CCA-2, CCA-3, CCB-1, CCB-2, CCB-5, CCD-1, CCD-3, CCD-4	9
Mushroom[c]	Phrae	MRA1-1, MRA1-2, MRA1-3, MRA2-2, MRA2-3, MRA2-4, MRA3-1, MRA3-2, MRA3-3, MRA3-4, MRA3-5	11
	Lamphun	MRB1-1, MRB1-2, MRB1-3, MRB1-4, MRB1-5, MRB2-1, MRB2-2, MRB2-3, MRB2-4, MRB3-2, MRB3-3, MRC2-1, MRC2-2, MRC2-3, MRD1-1, MRD1-2, MRD1-3, MRD1-4, MRD1-5, MRD1-6, MRE1-1, MRE1-2, MRE1-3	23
	Lampang	MRF1-1, MRE1-2, MRE1-3, MRE1-4, MRE1-6, MRG1-1, MRG1-2, MRG1-3, MRG1-4, MRG1-5, MRG1-6, MRG2-1, MRG2-2, MRG2-3, MRG3-1, MRG3-2, MRG3-3, MRH1-1, MRH1-2, MRH1-3, MRH1-4, MRH1-5, MRH1-6, MRH2-1, MRH2-2, MRH2-3, MRH2-4, MRH2-5, MRH3-1, MRH3-2, MRH3-3, MRH3-4, MRH3-5, MRI1-1, MRI1-3, MRI1-4, MRI1-5, MRI2-1, MRI2-2, MRI2-3, MRI2-4, MRI2-5, MRI3-1, MRI3-2, MRI3-3, MRI3-4, MRI3-5, MRJ1-1, MRJ1-2, MRJ1-3, MRJ1-4, MRJ1-5, MRJ2-1, MRJ2-2, MRJ2-3, MRJ2-4, MRJ3-1, MRJ3-2, MRJ3-3, MRK1-1, MRK1-2, MRK1-3, MRK1-4, MRK1-5, MRK2-1, MRK2-2, MRK2-3, MRK2-4, MRK3-1, MRK3-2, MRK3-3	71
Fermented tea leaves (*Miang*)[d]	Chiang Mai	FTLA-1, FTLA-2, FTLA-4, FTLA-5, FTLA-6, FTLB-1, FTLB-2	7
	Lampang	FTLC-1, FTLC-2, FTLC-3, FTLC-4, FTLD-1, FTLD-2, FTLD-3, FTLE-1, FTLE-2, FTLE-3, FTLE-4, FTLE-5	12

[a-e]See in Appendix D

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Table 4.1 Sample, Location, strain number and number of strain of LAB (Cont.)

Sample	Location	Strain no.	No. of strain
Fermented plant beverages	Chiang Mai	BGM-B1, BGM-B2, BGM-B3, BGM-B4, BGM-B5, BGM-B6, BGM-B7, BGM-B8, BGM-B9, BGM-B10, BGM-B11, BGM-B12, BGM-B13, BGM-B14, BGM-B15, BGM-B16, BGM-B17, BGM-M1, BGM-M2, BGM-M3, BGM-S1, BGM-S2, BGM-S4, BGM-S6, BGM-S9, BGM-S10, BGM-S11, TMT-C3	28
Fermented mushroom[e]	Nakhonrat-chasima	FM-B1, FM-B2, FM-B3, FM-B4, FM-B5, FM-B6, FM-B7, FM-B8, FM-B9, FM-B10, FM-B11, FM-B12, FM-B13, FM-B14, FM-B15, FM-B16, FM-B18, FM-B19, FM-B20, FM-B21, FM-B22, FM-B23, FM-B24, FM-B25, FM-B27, FM-S1, FM-S2, FM-S3, FM-S4, FM-S6, FM-S7, FM-S8, FM-S9, FM-S10, FM-S11, FM-S14, FM-S16, FM-S17, FM-S18, FM-S19, FM-S20, FM-S21, FM-S25, FM-S26,	44
Fermented rice noodle (*Khanom-jeen*)	Songkhla	HB3-3, HB3-4, SB1-2, SC2-1, SC3-4	5
	Nakhonrat-chasima	P1-1, P1-2A, P1-3, P2-2A, P2-4, P3-1, P4-1, P4-3, P4-6, P4-6A, P22-7, P22-7A, P22-9, P23-10, P23-13, P32-9, P41-3, P42-1, P42-1A, P42-4, P42-4A, P43-1, P43-1A, P43-01, N11-5, N11-7, N21-9	27
Total			**238**

[a-e]See in Appendix D

Lactic acid bacteria belonged to the genera *Lactobacillus, Leuconostoc, Pediococcus, Enterococcus* and *Lactococcus* that naturally relied on the surface of plant materials can spontaneously initiate the fermentation of fruits and vegetables (Mäki, 2004).

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4.2 Anti-*H. pylori* activity of LAB strains

All strains were screened for their antimicrobial activity against *H. pylori* by spot-on-lawn assay. Fifty-three strains showed the different levels of inhibition against *H. pylori* growth as shown in Table 4.2. There were 32 strains of LAB (MRA1-3, MRA3-4, MRA3-5, MRB1-1, MRB1-4, MRB3-3, MRK2-3, FTLC-4, BGM-S1, BGM-S2, BGM-S4, BGM-S6, BGM-S9, TMT-C3, FM-B6, FM-B7, FM-B8, FM-B16, FM-B18, FM-B20, FM-S16, FM-S19, FM-S25, HB3-3, P4-3, P22-7, P22-7A, P22-9, P23-13, P32-9, P42-1 and P42-1A) could inhibit the growth of *H. pylori* BK364, which were in the range of 5.8 to 12.8 mm. Seven strains (MRJ2-4, FTLA-1, FTLE-2, HB3-4, N21-9, SB1-2 and P43-1A) showed the suppression against *H. pylori* MS83, which contained the inhibition diameter in the range of 5.8 to 8.5 mm. Five strains (Ru20-1, MRI3-1, MRI3-2, FM-B9 and SC3-4) displayed the inhibition of *H. pylori* 2649, which were in the range of 5.5 to 6.5 mm. Two clinical *H. pylori* strains (strain MS83 and BK 364) were suppressed by 9 strains of LAB including MRK3-1, FTLB-1, FTLB-2, FTLC-1, FTLC-2, FTLE-3, FTLE-4, FTLE-5 and P43-01. The results demonstrated that *H. pylori* strain BK364 was the most sensitive whereas strain MS83 and 2649 were the less sensitive to CFSs collected from LAB. Among these LAB, strain FM-S16, HB3-4, MRI3-2 and P43-01 expressed the highest antimicrobial activity against *H. pylori* strain BK364 (12.8 mm), MS83 (8.5 mm) and 2649 (6.5 mm) and both MS83 (9.0 mm) and BK364 (11.0 mm), respectively. *H. pylori* infection remains a worldwide spread disease with a definite morbidity and mortality.

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Table 4.2 Anti-*H. pylori* activity and pH value of CFS collected from plant-derived LAB

No.	Strain no.	pH	Diameter of inhibition zone (mm) against *H. pylori* strain		
			2649	MS83	BK364
1	Ru20-1	5.34	5.8±1.0[a]	-	-
2	MRA1-3	3.85	-	-	6.5±0.6
3	MRA3-4	3.94	-	-	7.5±0.6
4	MRA3-5	4.39	-	-	7.5±0.6
5	MRB1-1	4.30	-	-	7.5±0.6
6	MRB1-4	4.36	-	-	7.5±0.6
7	MRB3-3	4.35	-	-	7.5±0.6
8	MRI3-1	4.44	5.8±1.0	-	-
9	MRI3-2	4.48	6.5±0.6	-	-
10	MRJ2-4	4.36	-	6.5±0.6	-
11	MRK2-3	3.82	-	-	5.8±1.0
12	MRK3-1	4.36	-	6.5±0.6	6.5±0.6
13	FTLA-1	3.89	-	6.5±0.7	-
14	FTLB-1	3.87	-	6.5±0.6	6.5±0.6
15	FTLB-2	4.27	-	6.5±0.6	6.5±0.6
16	FTLC-1	3.83	-	6.5±0.6	6.5±0.6
17	FTLC-2	3.85	-	6.5±0.6	6.5±0.6
18	FTLC-4	3.87	-	-	5.8±1.0
19	FTLE-2	4.39	-	6.5±0.6	-
20	FTLE-3	3.92	-	6.5±0.6	6.5±0.6
21	FTLE-4	4.22	-	6.5±0.6	6.5±0.6
22	FTLE-5	4.78	-	8.5±0.6	9.5±0.6
23	BGM-S1	4.01	-	-	9.5±0.6
24	BGM-S2	4.76	-	-	9.0±1.2
25	BGM-S4	4.41	-	-	11.5±0.6
26	BGM-S6	4.04	-	-	7.8±0.5
27	BGM-S9	4.37	-	-	11.5±0.6

-; no inhibition.

[a]Results indicated mean ± SD of two independent experiments.

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Table 4.2 Anti-*H. pylori* activity and pH value of CFS collected from plant-derived LAB (Cont.)

No.	Strain no.	pH	Diameter of inhibition zone (mm) against *H. pylori* strain		
			2649	MS83	BK364
28	TMT-C3	4.41	-	-	8.0±3.6
29	FM-B6	4.53	-	-	10.3±2.1
30	FM-B7	4.48	-	-	10.5±1.3
31	FM-B8	4.38	-	-	10.5±0.6
32	FM-B9	4.54	5.5±0.7	-	-
33	FM-B16	4.37	-	-	10.0±1.8
34	FM-B18	4.36	-	-	8.0±0.0
35	FM-B20	4.44	-	-	8.5±2.1
36	FM-S16	4.39	-	-	12.8±1.0
37	FM-S19	3.90	-	-	7.0±2.4
38	FM-S25	4.40	-	-	8.0±3.6
39	HB3-3	4.33	-	-	6.5±0.6
40	HB3-4	4.36	-	8.5±0.6	-
41	N21-9	4.69	-	6.0±1.2	-
42	SB1-2	3.95	-	5.8±1.0	-
43	SC3-4	4.68	5.8±1.0	-	-
44	P4-3	4.32	-	-	6.5±0.7
45	P22-7	4.32	-	-	11.5±0.6
46	P22-7A	4.41	-	-	9.3±1.0
47	P22-9	4.34	-	-	8.0±0.0
48	P23-13	4.37	-	-	6.3±1.0
49	P32-9	4.34	-	-	6.3±1.0
50	P42-1	4.37	-	-	7.0±1.8
51	P42-1A	4.37	-	-	8.3±1.3
52	P43-1A	4.40	-	7.0±2.4	-
53	P43-01	4.02	-	9.0±1.2	11.0±1.2

-; no inhibition.

[a]Results indicated mean ± SD of two independent experiments.

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Antibiotic-based therapy has been applied to treat patients who were infected with *H. pylori*. Unfortunately, no current therapy is able to achieve a 100% success rate due to the antibiotic resistance of *H. pylori* (Zullo *et al.*, 2012; Kaur and Kaur, 2016). Thus, the need for alternative or adjunctive therapy such as the use of probiotics for the *H. pylori* eradication has claimed by many researches (Hamilton-Miller, 2003; Canducci *et al.*, 2002). This study reported the anti-*H. pylori* activity of LAB strains isolated from several kinds of plant-based food products. Fifty-three LAB strains exhibited different spectrum of antimicrobial activity against clinical strains of *H. pylori*. The susceptibility of clinical *H. pylori* strains to CFSs collected from LAB seemed to be strain-dependent sensitivity. *H. pylori* 2649 isolated from patient with gastritis was more tolerant than strain MS83 and BK364. It was well known that antimicrobial metabolites produced by LAB included organic acids (mainly lactic and acetic acid), diacetyl, hydrogen peroxide, and bacteriocin (Ouwehand and Vesterlund, 2004). The LAB strains showed the anti-*H. pylori* activity were identified in further study based on their 16S rRNA gene sequence similarity.

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4.3 Identification of selected strains

From the total of 53 selected strains, they were 20 rods, 2 Tetrads, 13 cocci and 18 coccobacilli. They appeared singly, in pair, tetrad or in chain. All LAB strains were Gram-positive, catalase negative, non-motile and non-spore forming bacteria. They were identified and divided in to 16 groups based on their 16S rRNA gene

sequence similarity. The phylogenetic tree indicated their taxonomic position as shown in Figure 4.1-4.6 and 4.8-4.9. Phenotypic characteristics of a representative strain in each group was studied (Table 4.3). According to their 16S rRNA gene sequence, LAB strains were belonged to the genus *Lactobacillus* (*Lb.*), *Pediococcus* (*P.*), *Enterococcus (E.)*, *Leuconostoc (Ln.)*, *Lactococcus (Lc.)* and *Weissella (W.)*.

4.3.1 Group 1

Group 1 consisted of three rod-shaped strains (BGM-S1, BGM-S6 and FM-S19). They did not produce gas from glucose. These strains grew at 45 ºC, pH 3.5, 9.0 and in 6% NaCl. They did not contain *meso*-DAP in the cell wall and produced DL-lactic acid. They produced acid from tested carobohydrate but did not produce acid from glycerol, α- methyl- D- glucoside, rhamnose and xylose. A representative strain, BGM- S6 in group 1 (1,332 bp) was identified as *Lb. plantarum* subsp. *plantarum* due to its 100% similarity of 16S rRNA gene sequence to *Lb. plantarum* subsp. *plantarum* ATCC 14917^{T} (Bergey *et al.,* 1923) (Figure 4.1). Among lactobacilli, *Lb. plantarum* is the most abundant microorganism in fermented vegetables, plants, gastrointestinal tract (GIT) of humans, dairy products, etc (Siezen *et al.,* 2010). In the present study, *Lb. plantarum* could be isolated from fermented plant beverage (BGM-S1, BGM-S6) and fermented mushroom (FM-S19) collected in Thailand. *Lb. plantarum* have involved in plant materials. This specie had initiated the vegetable fermentations (Mäki, 2004). *Lb. plantarum* was isolated from fermented star fruit (*Averrhoa carambola*) beverage (Duangjitcharoen *et al.,* 2009). In addition, *Lb. plantarum* could be detected

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Table 4.3 Phenotypic characteristics of LAB strains

Characteristics	Group 1 (3)	Group 2 (10)	Group 3 (2)	Group 4 (1)	Group 5 (5)	Group 6(2)	Group 7 (1)	Group 8 (1)
Genera	LP	LS	PP	LR	LF	EF	EC	LG
Cell form	R	R	T	R	R	C	C	C
Gas from glucose	-	-	-	+	+	-	-	-
Arginine hydrolysis	-	-	-	-	+	+	+	+
Nitrate reduction	-	-	-	-	-	-	-	-
Starch hydrolysis	-	-	-	-	-	-	-	-
Slime formation	-	-	-	-	-	-	-	-
Growth at 45 °C	+	-	+	+	+	+	+	-
Growth at pH 3.5	+	+	+	+	+	-	-	-
pH 9.0	+	+	+	+	-	+	+	+
Growth in 6% NaCl	+	+	+	+	-	+	+	+
D-Amygdalin	+	+	+	+	-	+	+	+
L-Arabinose	+	+	+	-	+	+	+	+
Cellobiose	+	+	+	+	-	+	+	+
D-Galactose	+	+	+	+	+	+	+	+
Gluconate	+	+	-	+	+	-	+	+
Glycerol	-	-	-	-	-	-	-	+
Lactose	+	+	-	+	-	+	+	-
Maltose	+	+	+	+	+	+	+	+
D-Mannitol	+	+	-	+	-	+	+	+
D-Mannose	+	+	+	+	-	+	+	+
Melibiose	+	+	-	+	-	+	+	+
α-Methyl-D-glucoside	-	-	-	+	-	-	+	+
Raffinose	+	+	-	+	-	+	+	+
Rhamnose	-	-	-	-	-	-	-	-
Ribose	+	+	+	+	+	+	+	+
Salicin	+	+	+	+	-	+	+	+
Sorbitol	+	+	-	+	-	+	-	-
Sucrose	+	+	-	+	+	+	+	+
Trehalose	+	+	+	+	+	+	+	+
Xylose	-	-	+	+	-	-	+	+
meso-DAP	+	+	-	-	-	-	-	-
Isomer of lactic acid	DL	DL	DL	L	DL	L	L	L

LP, *Lactobacillus plantarum* subsp. *plantarum*; LS, *Lactobacillus pentosus*; PP, *Pediococcus pentosaceous*; LR, *Lactobacillus paracasei* subsp. *tolerans*; LF, *Lactobacillus fermentum*; EF, *Enterococcus faecium*; EC, *Enterococcus casseliflavus*; LG, *Lactococcus garvieae subsp. garvieae*; +, positive; –, negative; R, rods; C, cocci; T, Tetrads

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Table 4.3 Phenotypic characteristics of LAB strains (Cont.)

Characteristics	**Group 9 (3)**	**Group10 (2)**	**Group11 (2)**	**Group12 (2)**	**Group13 (1)**	**Group14 (6)**	**Group15 (11)**
Genera	LL	LT	LC	LM	WP	WC	WF
Cell form	C	C	C	C	CB	CB	CB
Gas from glucose	-	+	+	+	+	+	+
Arginine hydrolysis	+	-	-	-	-	-	-
Nitrate reduction	-	-	-	-	-	-	-
Starch hydrolysis	-	-	-	-	-	-	-
Slime formation	-	-	-	-	-	-	-
Growth at 45 °C	-	+	-	-	-	-	-
Growth at pH 3.5	-	+	-	-	-	-	-
pH 9.0	+	+	+	+	+	+	+
Growth in 6% NaCl	+	+	+	+	+	+	+
D-Amygdalin	+	+	+	-	-	-	-
L-Arabinose	+	+	-	-	+	+	-
Cellobiose	+	+	+	+	+	+	-
D-Galactose	+	+	+	+	+	-	+
Gluconate	-	+	+	+	+	+	+
Glycerol	-	-	-	-	-	-	-
Lactose	+	+	+	-	-	-	-
Maltose	+	+	-	+	+	+	+
D-Mannitol	+	-	-	-	-	-	-
D-Mannose	+	+	+	+	+	+	+
Melibiose	-	+	+	+	+	-	-
α-Methyl-D-glucoside	+	-	+	+	+	+	-
Raffinose	-	+	+	+	+	-	-
Rhamnose	-	-	+	-	-	-	-
Ribose	-	-	-	+	+	-	-
Salicin	+	+	+	-	-	-	-
Sorbitol	-	-	+	-	-	-	-
Sucrose	+	+	+	+	+	+	-
Trehalose	+	+	+	+	+	-	-
Xylose	-	+	-	+	+	+	+
meso-DAP	-	-	-	-	-	-	-
Isomer of lactic acid	L	D	D	D	D	DL	DL

LL, *Lactococcus lactis subsp. lactis*; LT, *Leuconostoc lactis*; LC, *Leuconostoc citreum*; LM, *Leuconostoc pseudomesenteroides*; WP, *Weissella paramesenteroides*; WC, *Weissella cibaria*; WF, *Weissella confusa*; +, positive; –, negative; w weak positive reactions; C, cocci; CB, Coccobacilli

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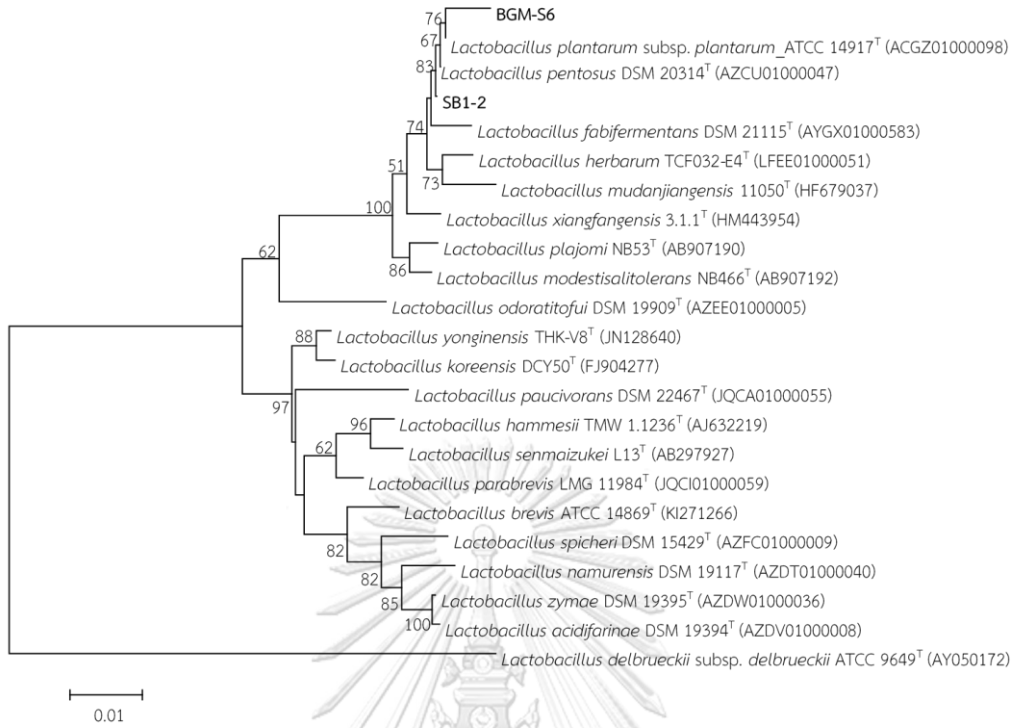

Figure 4.1 Neighbor-joining tree based on 16S rRNA gene sequences showing the relationship among the representative strains in group 1 and 2 and related species.

in several kinds of fermented vegetables including fermented ginger, broccoli, pak-sian, cabbage, mustard, beet etc (Swain *et al., 2014*).

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4.3.2 Group 2

Group 2 included ten rod-shaped strains which were MRA1-3, MRK2-3, FTLA-1, FTLB-1, FTLC-1, FTLC-2, FTLC-4, FTLE-3, FTLE-4 and SB1-2. They did not produce gas from glucose. They grew at pH 3.5, 9.0 and in 6% NaCl. No growth was observed at 45 ºC. They contained *meso*-DAP in the cell wall and produced DL-lactic acid. They produced acid from tested carbohydrates but did not produce acid from glycerol, α-methyl-D-glucoside and rhamnose (Table 4.3). Based on the 16S rRNA gene

sequence, a representative strain, SB1-2 in group 2 (1,399 bp) was identified as *Lb. pentosus* from its 99.93% similarity of 16S rRNA gene sequence to *Lb. pentosus* DSM 20314^{T} (Zanoni *et al.*, 1987) (Figure 4.1). *Lb. pentosus* strains were detected in mushroom (MRA1-3 and MRK2-3), fermented tea leaves (FTLA-1, FTLB-1, FTLC-1, FTLC-2, FTLC-4, FTLE-3 and FTLE-4) and Khanom-jeen (SB1-2). This species was associated with plants and had been found in Asian traditional fermented fruits and vegetables such as fermented eggplant, mustard, olive, cabbage, cucumber, celery and radish etc (Mäki, 2004; Swain *et al.*, 2014). Moreover, fermented tea leaves or Miang was found as a source of *Lb. pentosus* (Tanasupawat *et al.*, 2007). *Lb. plantarum*, *Lb. fermentum*, and *P. acidilacti* are the predominant bacteria in Khanom-jeen production (Tanasupawat and Komagata, 2001). *Lb. pentosus* isolated in this study also involved in Khanom-jeen product.

4.3.3 Group 3

Group 3 included two tetrad-shaped strains, MRA3-4 and MRA3-5. They did not produce gas from glucose. They grew pH 3.5, 9.0 and in 6% NaCl but did not grow at 45 ºC. Strains did not contain *meso*-DAP in the cell wall. They produced DL-lactic acid. All strains produced acid from tested carbohydrates except for glycerol, α-methyl-D-glucoside, rhamnose and xylose. (Table 4.3). A representative strain, MRA3-4 in group 3 (1,393 bp) was identified as *P. pentosaceus* based on its 100% similarity of 16S rRNA gene sequence to *P. pentosaceus* DSM 20336^{T} (Mees, 1934) (Figure 4.2). These strains were detected in mushroom. *P. pentosaceus* was the predominant LAB associated

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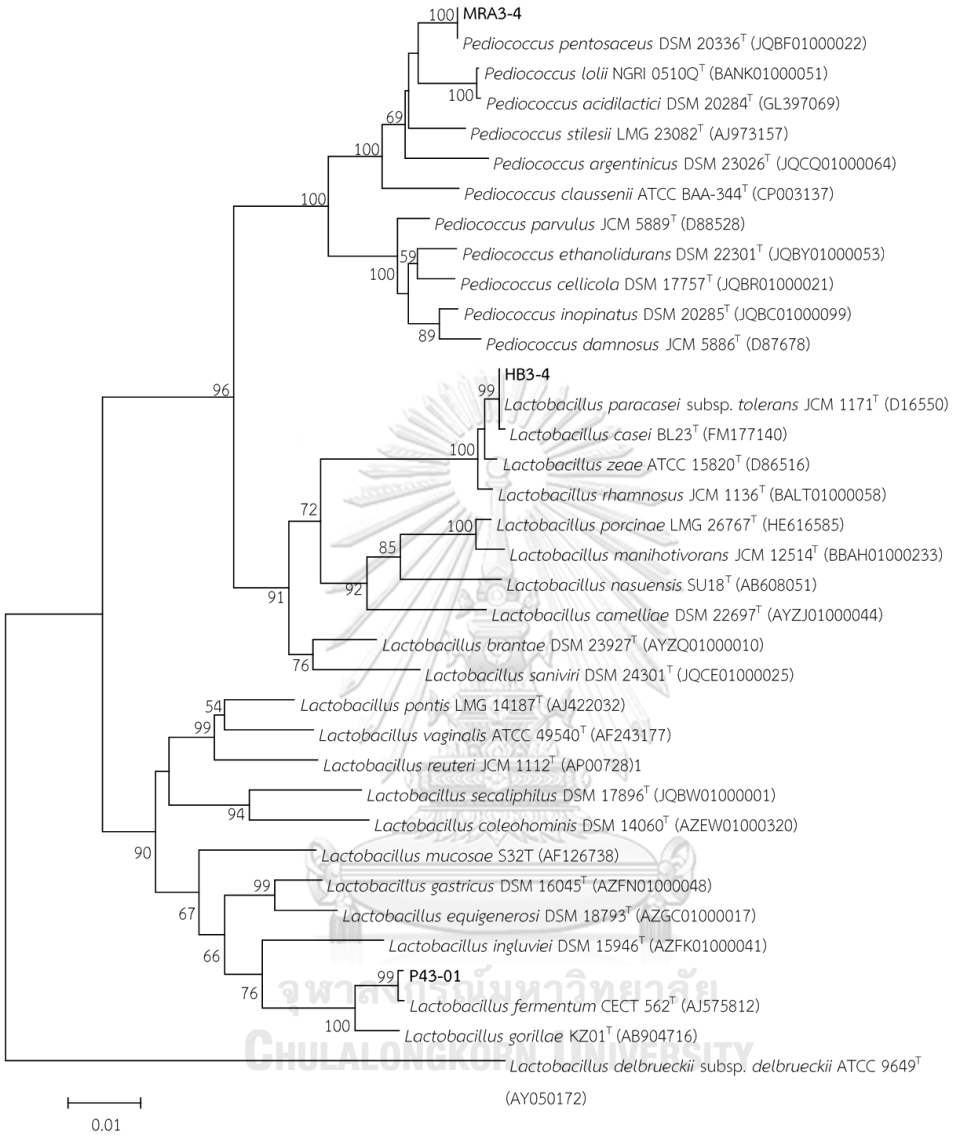

Figure 4.2 Neighbor-joining tree based on 16S rRNA gene sequences showing the relationship among the representative strains in group 3 to 5 and related species.

with fermented vegetables such as fermented bamboo shoot, pickle, mustard cabbage leaves, cucumber etc (Montet *et al.*, 2014; Swain *et al.*, 2014).

4.3.4 Group 4

Group 4 contained one rod-shaped strain, HB3-4. It produced gas from glucose. A strain grew at 45 ºC, pH 3.5, 9.0 and in 6% NaCl. It did not contain *meso*-DAP and produced L-lactic acid. A strain produced acid from D-amygdalin, cellobiose, D-galactose, gluconate, lactose, maltose, D-mannitol, D-mannose, melibiose, α-methyl-D-glucoside, raffinose, ribose, salicin, sorbitol, sucrose, trehalose, xylose (Table 4.3). According to the 16S rRNA gene sequence, this strain was identified as *Lb. paracasei* subsp. *tolerans* from its 99.57% similarity sequence (1,403 bp) to *Lb. paracasei* subsp. *tolerans* JCM 1171^{T} (Collins *et al.*, 1989) (Figure 4.2). A strain HB3-4 was isolated from Khanom-jeen. LAB that involved in Khanom-jeen process were *Lb. plantarum*, *Lb. fermentum*, and *P. acidilactici* (Tanasupawat and Komagata, 2001). In the current study, *Lb. paracasei* subsp. *tolerans* was also found in fermented rice noodle.

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4.3.5 Group 5

Group 5 consisted of five rod-shaped strains including HB3-3, P22-7, P22-7A, P22-9 and P43-01. All strains produced gas from glucose. They could hydrolyze arginine. They grew at 45 ºC and pH 3.5 but did not grow at pH 9.0 and in 6% NaCl. They did not contain *meso*-DAP in the cell wall and produced DL-lactic acid. Tested strains produced acid from L-arabinose, D-galactose, gluconate, maltose, ribose, sucrose and trehalose (Table 4.3). A representative strain, P43-01 in group 5 was identified as *Lb. fermentum* from its 99.86% similarity of 16S rRNA gene sequence

(1,395 bp) to *Lb. fermentum* CECT 562^{T} (Kandler and Weiss, 1986) (Figure 4.2). The strains in this group was isolated from Khanom-jeen (HB3-3, P22-7, P22-7A, P22-9 and P43-01). *Lb. fermentum* has been isolated from fruits such as pineapple, cherry, soursop, Mango and strawberry (Garcia *et al.*, 2016). *Lb. plantarum*, *Lb. fermentum*, and *P. acidilactici* were the major bacteria associated with Khanom-jeen production (Tanasupawat and Komagata, 2001). Lu *et al.*, 2008 revealed that *Lb. fermentum* found at the beginning and dominated during the rice noodles fermentation. Moreover, *Lb. fermentum* was predominated during the fermentation process of fermented rice noodles produced from different factories in China (Li *et al.*, 2015).

4.3.6 Group 6

Group 6 included two coccal strains, N21-9 and SC3-4. They did not produced gas from glucose. They showed positive for hydrolysis of arginine. Growth was observed at 45 ºC, pH 9.0 and in 6% NaCl. They did not contain *meso*-DAP in the cell wall and they produced only L-lactic acid. The strains produced acid from D-amygdalin, L-arabinose, cellobiose, D-galactose, lactose, maltose, D-mannitol, D-mannose, melibiose, raffinose, ribose, salicin, sorbitol, sucrose and trehalose (Table 4.3). Based on 16S rRNA gene sequence, a representative strain, N21-9 (1,373 bp) was identified as *E. faecium* from its 100% similarity of sequence to *E. faecium* CGMCC 1.2136^{T} (Schleifer and Kilpper-balz, 1984) (Figure 4.3). These strains were isolated from Khanom-jeen. The dominant microflora in Khanom-jeen process were *Lb. plantarum*, *Lb. fermentum*, and *P. acidilactici* (Tanasupawat and Komagata, 2001). *E. faecium* could also be

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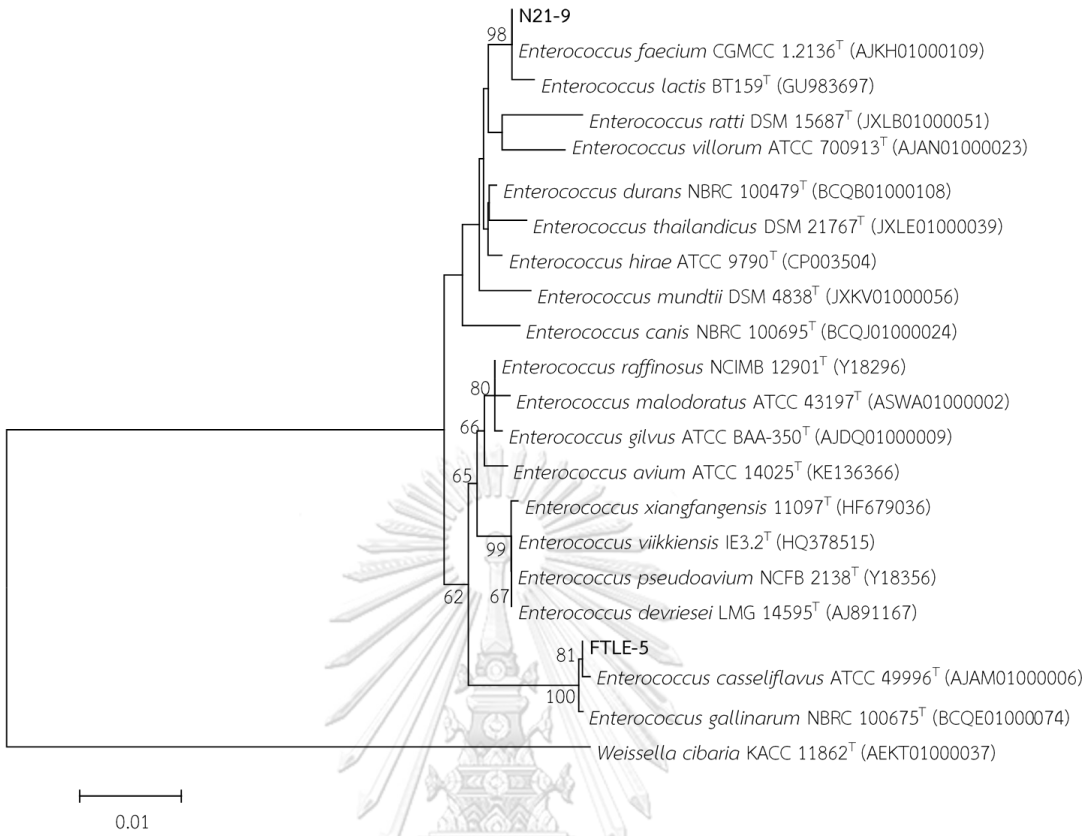

Figure 4.3 Neighbor-joining tree based on 16S rRNA gene sequences showing the relationship among the representative strains in group 6 and 7 and related species.

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detected in Khanom-jeen product. Moreover, *E. faecium* could be found in many plant materials (Mäki, 2004).

4.3.7 Group 7

Group 7 comprised of one coccal strain, FTLE-5. A strain did not produce gas from glucose. It could hydrolyze arginine. Strain grew at 45 °C, pH 9.0 and in 6% NaCl. It did not contain *meso*-DAP in the cell wall. A strain produced only L-lactic acid. It produced acid from tested carbohydrates but did not produce from glycerol,

rhamnose and sorbitol (Table 4.3). A strain was identified as *E. casseliflavus* from 99.93% similarity based on its 16S rRNA gene sequence (1376 bp) to *E. casseliflavus* NBRC 100675^{T} (Collins *et al.*, 1984) (Figure 4.3). A strain in this group was isolated from fermented tea leaves. There is no study reported on the detection of *E. casseliflavus* in fermented tea leaves. *E. casseliflavus* was found in fermented cucumber (Swain *et al.*, 2014).

4.3.8 Group 8

Group 8 contained one coccal strain, BGM-S2. It did not produce gas from glucose. A strain provided positive result in arginine hydrolysis testing. Growth was observed at pH 9.0 and in 6% NaCl. A strain did not contain *meso*-DAP in the cell wall. It produced L-lactic acid. It produced acid from tested carbohydrates except for lactose, rhamnose and sorbitol (Table 4.3). According to 16S rRNA gene sequence, this strain was identified as *Lc. garvieae* subsp. *garvieae* from its 99.93% similarity of sequence (1,339 bp) to *Lc. garvieae* subsp. *garvieae* ATCC 49156^{T} (Varsha and Nampoothiri, 2016) (Figure 4.4). A strain in this group was isolated from fermented plant beverages. *Lc. garvieae* subsp. *garvieae* has been isolated from a guar dung (Varsha and Nampoothiri, 2016). There is no study reported on the detection of this species in plants and their fermented products.

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4.3.9 Group 9

Group 9 included three coccal strains which were P43-1A, FTLE-2 and MRB1-4. All strains did not produce gas from glucose. They could hydrolyze arginine. They grew

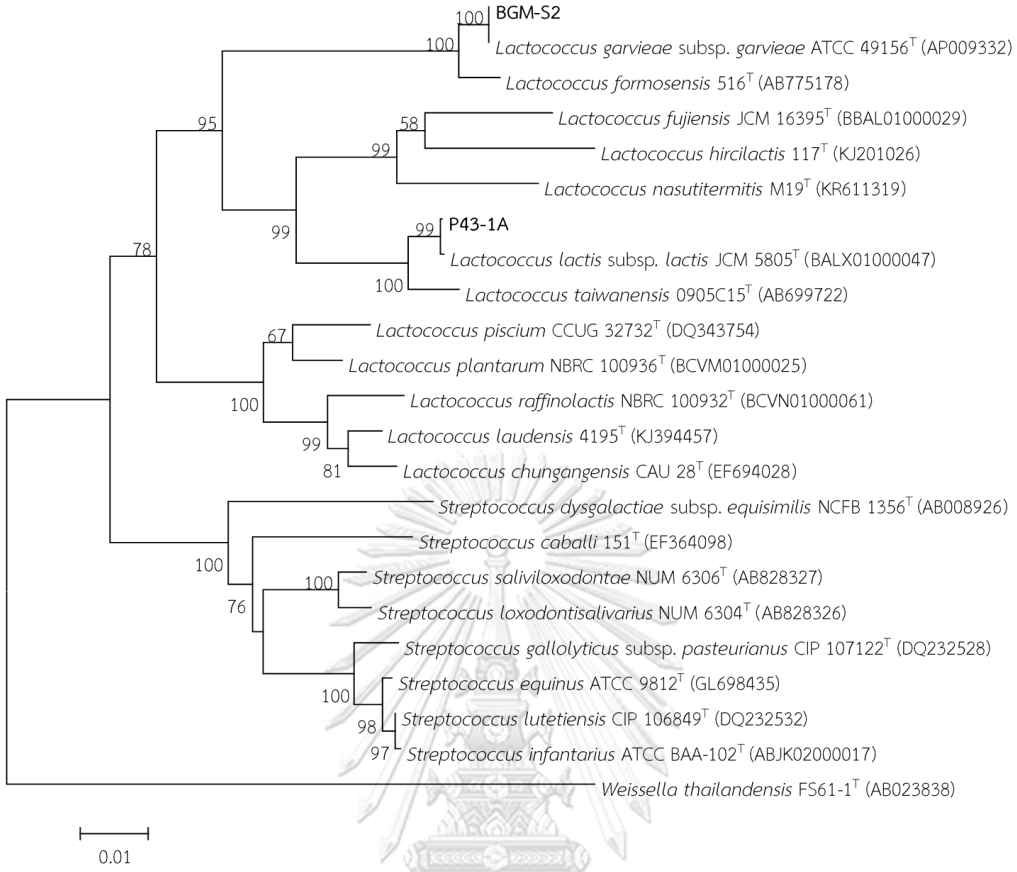

Figure 4.4 Neighbor-joining tree based on 16S rRNA gene sequences showing the relationship among the representative strains in group 8 and 9 and related species.

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at pH 9.0 and in 6% NaCl but did not at pH 3.5 and 45 ºC. They did not contain *meso*-DAP in the cell wall and produced only L- lactic acid. They produced acid from D- amygdalin, L- arabinose, cellobiose, D- galactose, lactose, maltose, D- mannitol, D-mannose, α-methyl-D-glucoside, salicin, sucrose and trehalose (Table 4.3). Based on the 16S rRNA gene sequence, a representative strain, P43-1A (1,359 bp) was identified as *Lc. lactis* subsp. *lactis* from its 99.93% similarity of sequence to *Lc. lactis* subsp. *lactis* JCM 5805^T (Schleifer *et al.*, 1985) (Figure 4.4). The strains in present study were

isolated from Khanom-jeen (P43-1A), Miang (FTLE-2) and mushroom (MRB1-4). *Lc. lactis* has involved in plant materials (Mäki, 2004). *Lc. lactis* subsp. *lactis* has been found in fruit fermentation such as fermented plum and cucumber (Swain *et al.*, 2014).

4.3.10 Group 10

Group 10 consisted of two coccal strains (P32-9 and MRB1-1). All strains produced gas from glucose. They grew at 45 ºC, pH 3.5, 9.0 and in 6% NaCl. They did not contain *meso*-DAP in the cell wall and produced only D-lactic acid. They produced acid from tested carbohydrate except for glycerol, D-mannitol, α-methyl-D-glucoside, rhamnose, ribose and sorbitol (Table 4.3). A representative strain, P32-9 (1,395 bp) was identified as *Ln. lactis* due to its 99.78% similarity of 16S rRNA gene sequence to *Ln. lactis* DSM 20202^{T} (Vancanneyt *et al.*, 2006) (Figure 4.5). The strains in this group were isolated from Khanom-jeen (P32-9) and mushroom (MRB1-1). *Ln. lactis* has been found in Mifen, a traditional fermented rice noodles of China (Lu *et al.*, 2008). *Ln. lactis* was associated with the fermentation of traditional cucumber fermentation (Swain *et al.*, 2014). However, no research reported on the detection of this species in mushroom.

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4.3.11 Group 11

Group 11 contained two coccal strains, P42-1 and P42-1A. All strains produced gas from glucose. They grew at pH 9.0 and in 6% NaCl. They did not have *meso*-DAP in the cell wall and produced only D-lactic acid. They produced acid from tested carbohydrates except for L-arabinose, glycerol, D-mannitol, ribose and xylose

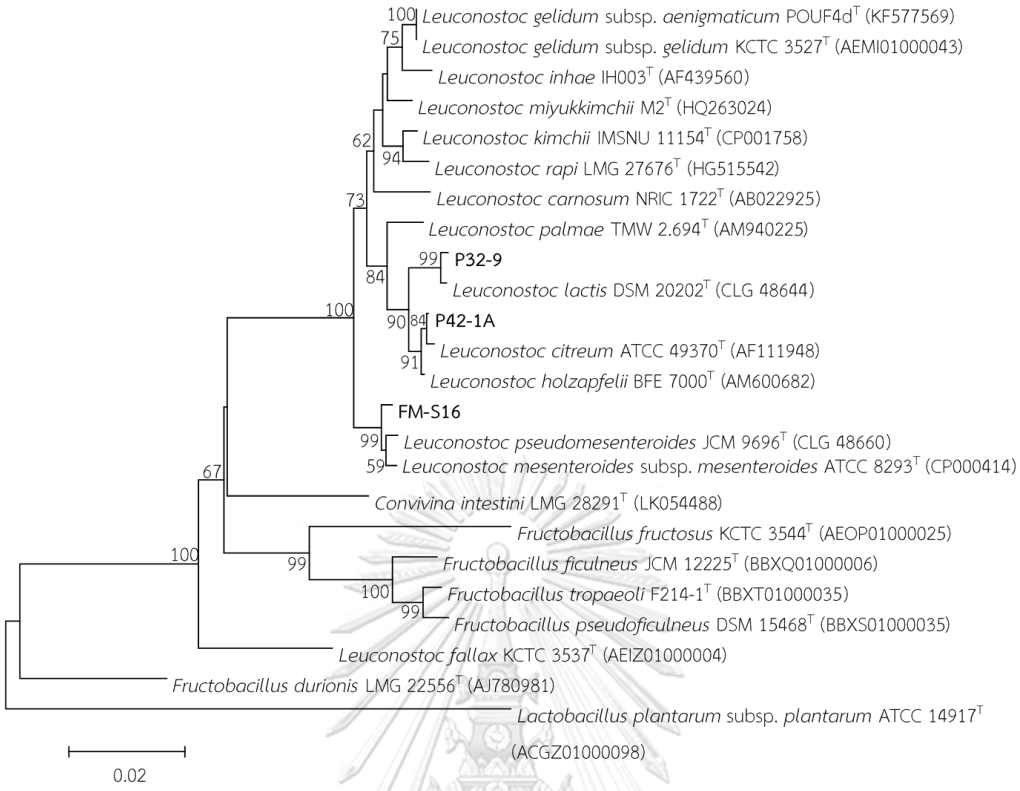

Figure 4.5 Neighbor-joining tree based on 16S rRNA gene sequences showing the relationship among the representative strains in group 10 to 12 and related species.

(Table 4.3). A representative strain, P42-1A (1361 bp) was identified as *Ln. citreum* based on its 99.70% similarity of 16S rRNA gene sequence to *Ln. citreum* ATCC 49370^T (Farrow *et al.*, 1989) (Figure 4.5). The strains in this group were isolated from Khanom-jeen. Tanasupawat and Komagata, 2001 suggested that LAB contributing to Khanom-jeen processing included *Lb. plantarum*, *Lb. fermentum*, and *P. acidilactici*. This study revealed that *Ln. citreum* was also isolated from Khanom-jeen.

4.3.12 Group 12

Group 12 comprised of two coccal strains which were BGM-S4 and FM-S16. All strains produced gas from glucose. Growth was observed at pH 9.0 and in 6% NaCl. They did not contain meso-DAP in the cell wall. All strains produced only D-lactic acid. They produced acid from tested carbohydrates but did not from D- amygdalin, L- arabinose, glycerol, lactose, D- mannitol, rhamnose, salicin and sorbitol (Table 4.3). Based on the 16S rRNA gene sequence, a representative strain, FM-S16 (1,470 bp) was identified as *Ln. pseudomesenteroides* from its 99.66% similarity of sequence to *Ln. pseudomesenteroides* JCM 9696^{T} (Farrow *et al.*, 1989) (Figure 4.5). *Ln. pseudomesenteroides* strain BGM- S4 and FM- S16 were isolated from fermented plant beverage and fermented mushroom, respectively. *Leuconostoc* was the one of the genus in LAB group that dominated in vegetable fermentation (Mäki, 2004). Moreover, *Ln. pseudomesenteroides* seem to be responsible for kimchi fermentation (Swain *et al.*, 2014).

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4.3.13 Group 13

Group 13 included one coccobacillary strain, BGM- S9. A strain could produce gas from glucose. This strain grew at pH 9.0 and in 6% NaCl. It did not has *meso*-DAP. A strain produced D- lactic acid. It produced acid from L- arabinose, cellobiose, D- galactose, gluconate, maltose, D- mannose, melibiose, α- methyl- D- glucoside, raffinose, ribose, sucrose, trehalose and xylose (Table 4.3). This strain (1,382 bp) was identified as *W. paramesenteroides* based on its 99.78% similarity of 16S rRNA gene

sequence to *W. paramesenteroides* ATCC 33313^T (Collins *et al.*, 1993) (Figure 4.6). *W. paramesenteroides* BGM-S9 was isolated from fermented plant beverage. In addition, *W. paramesenteroides* was found to be the dominant species in various fermented vegetables including Yan-dong-gua (fermented wax gourd), Pobuzihi (fermented cummingcordia), Yan-tsai-shin (fermented Broccoli) in Taiwan and Yan-taozih (pickled peach) in China (Swain *et al.*, 2014).

4.3.14 Group 14

Group 14 consisted of six coccobacillary strains (FM-S25, FTLB-2, MRK3-1, MRJ2-4, MRB3-3, FM-B16). All strains produced gas from glucose. They grew at pH 9.0 and in 6% NaCl. They did not contain *meso*-DAP. The strains produced DL-lactic acid. They produced acid from L-arabinose, cellobiose, gluconate, maltose, D-mannose, α-methyl-D-glucoside, sucrose and xylose (Table 4.3). A representative strain, FM-B16 (1,370 bp) was identified as *W. cibaria* from its 99.78% similarity of 16S rRNA gene sequence to *W. cibaria* KACC 11862^T (Björkroth *et al.*, 2002) (Figure 4.6). These strains were isolated from fermented mushroom (FM-S25 and FM-B16), mushroom (MRK3-1, MRJ2-4 and MRB3-3) and Miang (FTLB-2). Swain *et al.*, 2014 suggested that *W. cibaria* was the culture responsible for fermentation of plants including Yan-dong-gua, Pobuzihi, Yan-jiang (fermented ginger), Yan-tsai-shin, Jiang-gua (fermented cucumber) and Yan-taozih.

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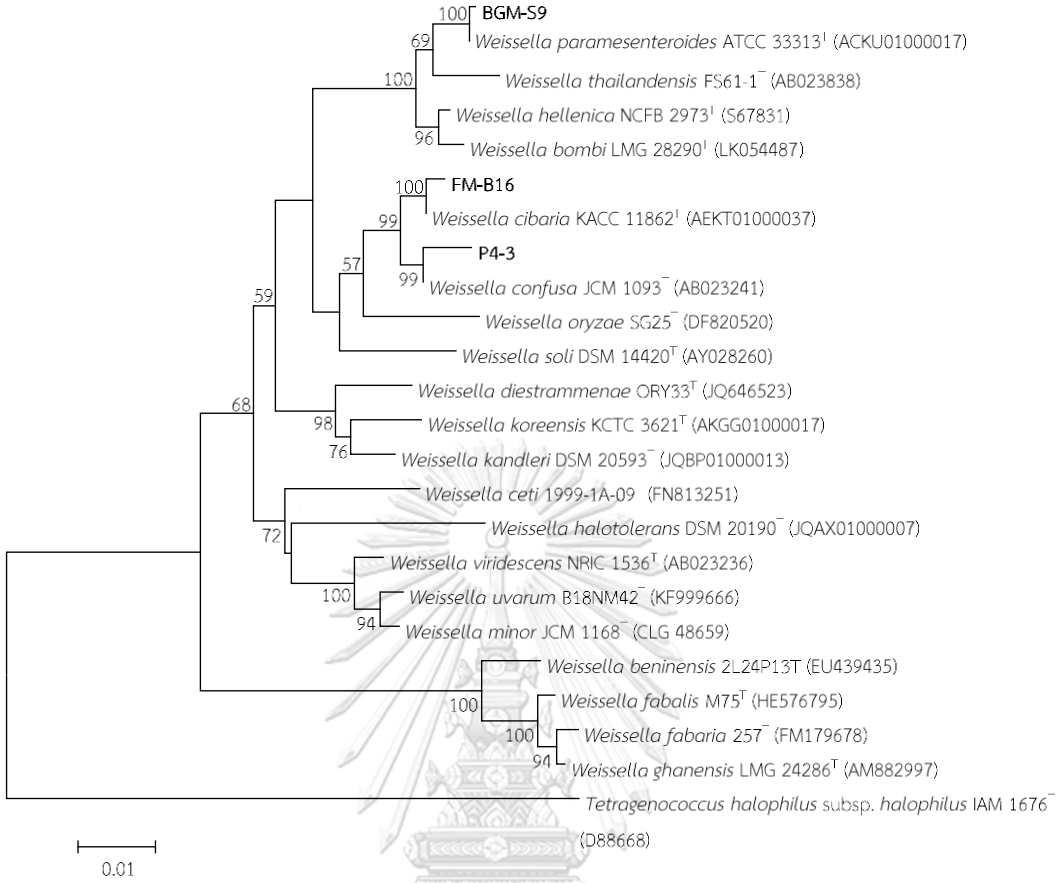

Figure 4.6 Neighbor-joining tree based on 16S rRNA gene sequences showing the relationship among the representative strains in group 13 to 15 and related species.

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4.3.15 Group 15

Group 15 contained eleven coccobacillary strains which were FM-B7, FM-B8, FM-B18, TMT-C3, MRI3-2, FM-B6, FM-B9, FM-B20, P4-3, MRI3-1 and P23-13. All strains produced gas from glucose. They grew at pH 9.0 and in 6% NaCl. They did not contain *meso*-DAP in the cell wall. They produced DL-lactic acid. They produced acid from D-galactose, gluconate, maltose, D-mannose and xylose. Based on the 16S rRNA sequence, a representative strain P4-3 (1,380 bp) was identified as *W. confusa* from its

100% similarity of sequence to *W. confusa* JCM 1093^{T} (Collins *et al.*, 1993) (Figure 4.6). In this study, *W. confusa* was isolated from fermented mushroom (FM-B6, FM-B7, FM-B8, FM-B9, FM-B18 and FM-B20), fermented plant beverage (TMT-C3), mushroom (MRI3-1 and MRI3-2) and Khanom-jeen (P23-13). *W. confusa* has been found in fermented plant products such as kimchi (Swain *et al.*, 2014).

4.3.16 Group 16

Group 16 included one rod-shaped strains which was Ru20-1. Cells of strain Ru20-1 were a Gram-stain-positive, non-spore-forming, non-motile and facultatively anaerobic rods (0.35–0.47 × 0.64–2.55 μm) as shown in Figure 4.7. Colonies were circular, smooth, low-convex and white with entire margins. Catalase was negative. Strain Ru20-1 produced gas from glucose and fermented glucose heterofermentatively producing both D- and L-isomers of lactic acid. This strain could produce diacetyl. The strain grew well at 15–40 ºC with optimum temperature at 30–37 ºC and could grow in the range of pH 4.5–7.0 with optimum growth at pH 6.5. In addition, growth was observed when strain Ru20-1 was cultivated in medium with 1–7% (w/v) NaCl. However, the strain could not grow at temperature above 40 ºC, pH 3.0–4.0 or 7.5–9.0 or with 9% (w/v) NaCl.

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On the basis of 16S rRNA gene sequence analyses, strain Ru20-1 (1475 bp) was closely related to *Lb. lindneri* LMG 14528^{T} (96.8%), *Lb. sanfranciscensis* NRIC 1548^{T} (95.4 %) and *Lb. florum* NRIC 0771^{T} (95.2 %), respectively. The phylogenetic analysis based on 16S rRNA gene and *pheS* gene sequences showed that strain Ru20-1^{T}

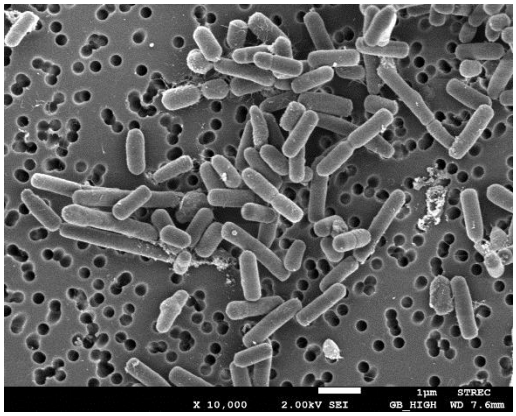

Figure 4.7 Scanning electron micrograph of *Lactobacillus ixorae* Ru20-1^T grown on MRS broth at 30 °C for 24 h. Bar 1 m.

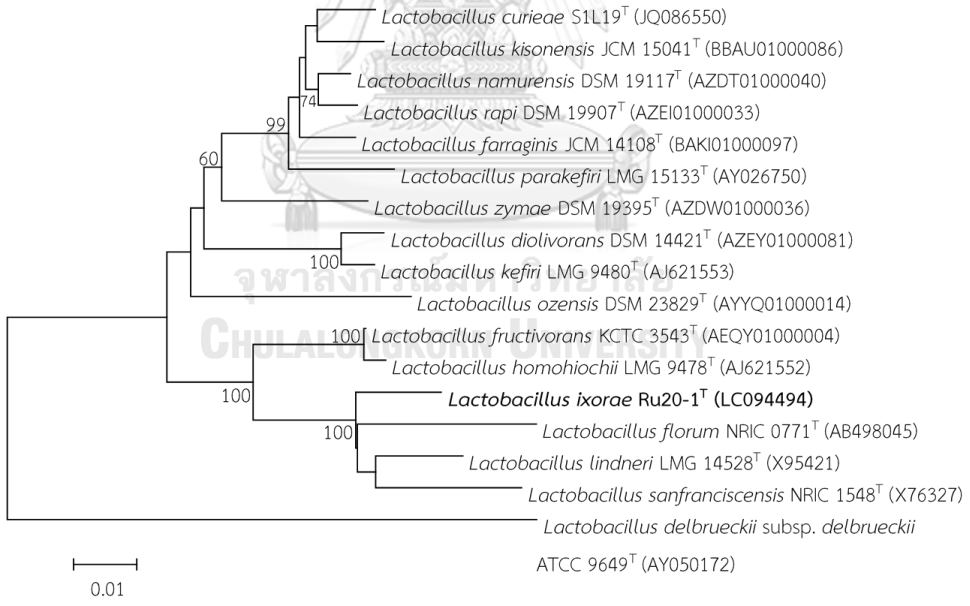

Figure 4.8 Neighbor-joining tree based on 16S rRNA gene sequences showing the relationship among the strain Ru20-1^T and related species of the genus *Lactobacillus*.

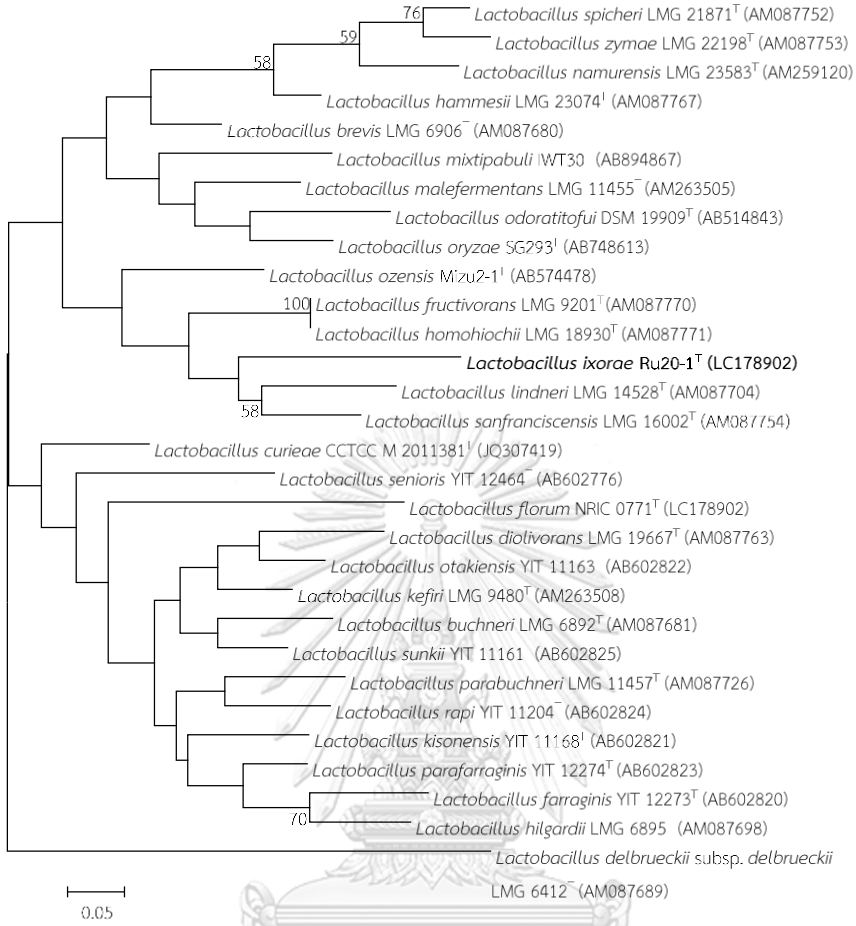

Figure 4.9 Maximum-likelihood tree based on *pheS* gene sequences showing the relationship between strain Ru20-1^T and related species of the genus *Lactobacillus.*

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and the closely related species formed a separate branch within the clade of the genus *Lactobacillus* as shown in Figure 4.8 and 4.9.

The DNA G+C content of strain Ru20-1^T was 47.8 mol% (Table 4.4), which was in the range of 32–55 mol% of the members of the genus *Lactobacillus* (Hammes and Hertel, 2009) and was higher than those of the closely related type strains. The cell-wall peptidoglycan type was L-Lys–D-Asp. Based on its morphological,

Table 4.4 Differential characteristics of strain Ru20-1^T and related type strains

Characteristic	**1**[a]	**2**	**3**	**4**
Temperature range for growth (°C)	15-40	15-30	15-30	15-30
Optimum temperature for growth (°C)	30-37	20-25	20-25	20-25
pH range for growth	4.5-6.5	4.5-6.5	4.5-6.5	5.0-6.5
Optimum pH for growth	6.5	4.5-5.5	5.5-6.5	6.5
NaCl concentration range for growth (%)	1-7	1	1-2	1-5
Optimum concentration of NaCl (%)	1-2	1	1-2	1-2
Diacetyl production	+	-	-	-
Acid production from				
D-Fructose	+	+	-	+
Gluconate	w	-	-	w
D-Maltose	-	+	+	-
D-Mannitol	+	-	-	-
DNA G+C content (mol%)	47.8	37.3	37.4	43.9

[a]Strains: 1, Ru20-1^T; 2, *Lb. lindneri* LMG 14528^T; 3, *Lb. sanfranciscensis* NRIC 1548^T; 4, *Lb. florum* NRIC 0771^T.
+, positive; w, weakly positive; –, negative reaction.

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chemotaxonomic and phylogenetic characteristics, the strain was determined to belong to the genus *Lactobacillus* (Hammes and Hertel, 2009). Strain Ru20-1^T contained $C_{18:1}$ ω9c (29.3 %), $C_{20:0}$ (13.0 %), $C_{20:1}$ ω9c (10.1 %) and summed feature 7 (unknown 18.846 and/or $C_{19:1}$ ω6c and/or $C_{19:0}$ cyclo) (29.9 %) as the major cellular fatty acids (Table 4.5). Strain Ru20-1^T showed differences in the amounts of fatty acids from the other type strains; for example, $C_{19:0}$ cyclo ω8c was not found in strain

Table 4.5 Cellular fatty acid composition of strains Ru20-1^T and related type strains

Fatty acid	1[a]	2	3	4
$C_{14:0}$	0.2	0.4	-	-
$C_{16:0}$	3.4	3.7	15.4	5.8
$C_{18:0}$	9.0	8.4	1.6	7.7
$C_{20:0}$	13.0	6.5	-	5.2
iso-$C_{19:0}$	0.8	0.4	1.6	0.3
$C_{18:1}$ ω9c	29.3	7.5	68.2	9.3
$C_{20:1}$ ω7c	0.5	19.5	-	0.6
$C_{20:1}$ ω9c	10.1	0.9	8.1	20.1
$C_{19:0}$ cycloω8c	-	28.0	-	-
Summed Feature 3[b]	0.5	1.0	0.4	0.2
Summed Feature 4[c]	0.3	0.5	0.6	0.4
Summed Feature 7[d]	29.9	11.1	-	49.4
Summed Feature 8[e]	3.1	12.0	4.0	1.0

[a]Strains: 1, Ru20-1^T; 2, *Lb. lindneri* LMG 14528^T; 3, *Lb. sanfranciscensis* NRIC 1548^T; 4, *Lb. florum* NRIC 0771^T. All data are shown as a percentage of the total fatty acids. -, Not detected.

[b]Summed Feature 3, $C_{16:1}$ ω7c and/or $_{16:1}$ω6c.

[c]Summed Feature 4, $C_{17:1}$ iso I and/ or $C_{17:1}$ anteiso B.

[d]Summed Feature 7, unknown fatty acid at retention time18.846 and /or $C_{19:1}$ ω6c and/ or $C_{19:0}$ cyclo.

[e]Summed Feature 8, $C_{18:1}$ ω7c or $C_{18:1}$ ω6c.

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Ru20-1^T, *Lb. sanfranciscensis* NRIC 1548^T (=ATCC 27651^T) and *Lb. florum* NRIC 0771^T but was detected only in *Lb. lindneri* LMG 14528^T (=DSM 20690^T). Summed feature 7, $C_{20:0}$ and $C_{20:1}$ ω7c were found in Ru20-1^T, *Lb. lindneri* LMG 14528^T and *Lb. florum* NRIC 0771^T but not detected in *Lb. sanfranciscensis* NRIC 1548^T.

The details of physiological and biochemical characteristics of strain Ru20-1 compared with those of closely related species are given in the species description as

showed in Table 4.4. Strain Ru20-1^{T} could be differentiated from *Lb. lindneri* LMG 14528^{T}, *Lb. sanfranciscensis* NRIC 1548^{T} and *Lb. florum* NRIC 0771^{T} by its growth at 40 ºC, optimum growth at pH 6.5, growth with 7% NaCl, the production of diacetyl, acid production from fructose, gluconate and D-mannitol, the DNA G+C content and the presence of $C_{18:1}$ ω9c as the major fatty acids, as shown in Table 4.4 and 4.5. Based on the phenotypic, chemotaxonomic and genotypic data, strain Ru20-1^{T} represents a novel species of the genus *Lactobacillus*, for which the name *Lactobacillus ixorae* sp. nov. is proposed (Techo *et al.*, 2016).

4.4 Probiotic properties of selected strain

4.4.1 Adherence ability

Adherence ability of LAB onto the epithelial cells was the key characteristic for colonization and the exclusion of bacterial pathogen in digestive tract. Therefore, the adherence capacity was the main criteria which has to be screened for selection of probiotic strains (Blum *et al.*, 1999; Ren *et al.*, 2012; Tareb *et al.*, 2013). In this study, AGS cells was used to screened for the adherence ability of selected LAB. As shown in Table 4.6, nine selected strains containing high anti-*H. pylori* activity were selected to determine their adherence ability. The adherence percentage to AGS cells of selected LAB strains was presented in Figure 4.10a. *Ln. pseudomesenteroides* BGM-S4 (20. 4%) exhibited the highest adherence rate and followed by *Ln. pseudomesenteroides* FM-S16 (12.9%). Adherence rate to AGS cells

Table 4.6 Nine LAB strains containing high anti-*H. pylori* activity

No.	Strain	Diameter of inhibition zone (mm) against *H. pylori*		
		2649	MS83	BK364
1	*Ln. pseudomesenteroides* FM-S16	-	-	12.8±1.0[a]
2	*Ln. pseudomesenteroides* BGM-S4	-	-	11.5±0.6
3	*W. paramesenteroides* BGM-S9	-	-	11.5±0.6
4	*Lb. fermentum* P22-7	-	-	11.5±0.6
5	*Lb. paracasei* subsp. *tolerans* HB3-4	-	8.5±0.6	-
6	*Lc. lactis* subsp. *lactis* P43-1A	-	7.0±2.4	-
7	*W. confusa* MRI3-2	6.5±0.6	-	-
8	*Lb. fermentum* P43-01	-	9.0±1.2	11.0±1.2
9	*En. casseliflavus* FTLE-5	-	8.5±0.6	9.5±0.6

-; no inhibition.

[a]Results indicated mean ± SD of two independent experiments.

of *Ln. pseudomesenteroides* was significantly higher than those of *W. confusa* MRI3-2 (3.6%), *W. paramesenteroides* (2.9%), *Lb. paracasei* subsp. *tolerans* (2.6%), *Lb. fermentum* P22-7 (1.6%), *Lb. fermentum* P43-01 (1.5%), *Lc. lactis* subsp. *lactis* P43-1A (0.5%) and *En. casselliflavus* FTLE-5 (0.2%).

The LAB adherence on AGS cells analyzed by the microscopic enumeration revealed a similar trend with those determined by the cultural enumeration. (Figure 4.10b). *Ln. pseudomesenteroides* BGM-S4 had the greatest adherence capability (26.4 bacteria per cell) followed by *Ln. pseudomesenteroides* FM-S16 (18.9 bacteria per cell).

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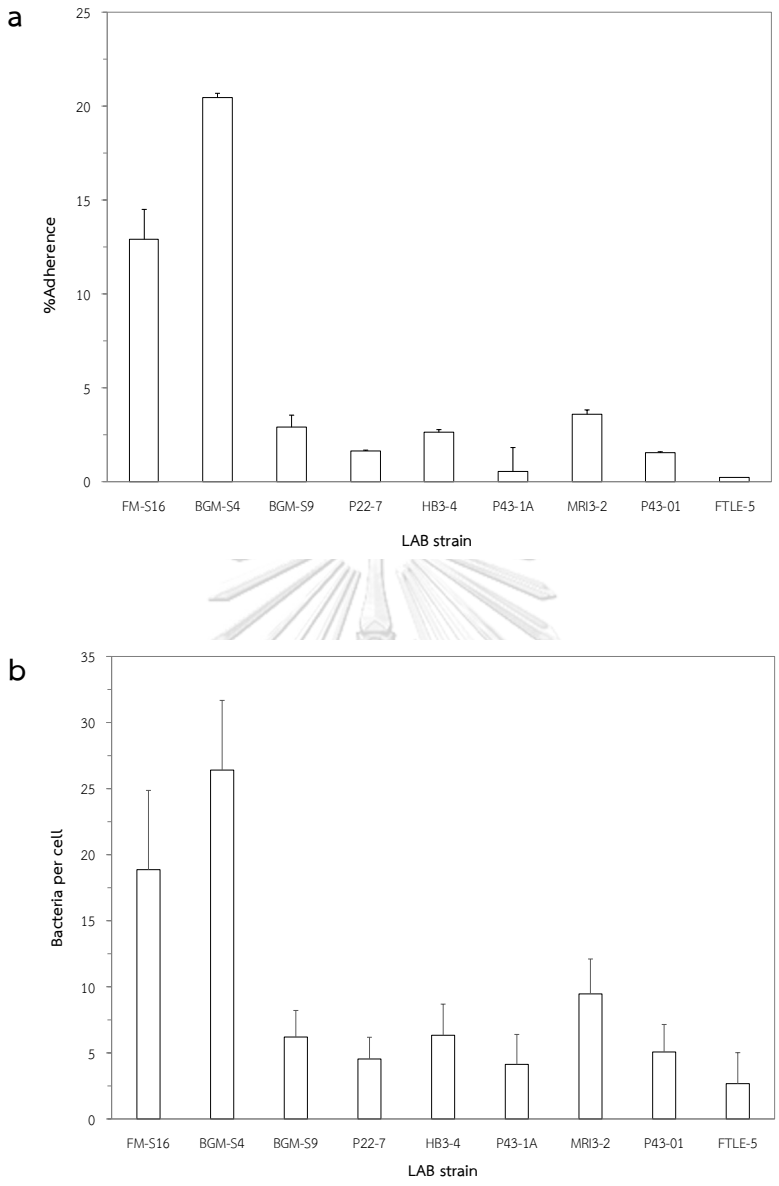

Figure 4.10 Adhesion rate of bacteria to AGS human gastric cell lines determined by cultural enumeration **(a)** and microscopic enumeration **(b).**

The lower capabilities of adherence to AGS cell lines were found in *W. confusa* MRI3-2 (9.5 bacteria per cell), *Lb. paracasei* subsp. *tolerans* (6.3 bacteria per cell), *W. paramesenteroides* BGM-S4 (6.2 bacteria per cell), *Lb. fermentum* P43-01

(5.1 bacteria per cell), *Lb. fermentum* P22-7 (4.5 bacteria per cell), *Lc. lactis* subsp. *lactis* P43-1A (4.1 bacteria per cell) and *En. gallinarum* FTLE-5 (2.7 bacteria per cell).

The adhesion of probiotic strains varies among strains, depending on the cell surface properties such as hydrophobicity and extracellular protein profiles (Botes *et al.*, 2008). There are three gene encoding for a putative mucus binding protein, a serine-rich protein and a putative collagen-adhesion protein are involved the adhesion ability of *Leuconostoc* (Johansson *et al.*, 2011). Thus, *Leuconostoc* spp. expressed the greater adherence ability than tested other strains.

4.4.2 Acid and bile tolerance

To evaluate their possible use in the treatment of *H. pylori* infection, the probiotic properties including acid and bile tolerance were carried out. According to Table 4.6, *Ln. pseudomesenteroides* FM-S16 showed the highest activity against only one strain of *H. pylori*, strain BK364. On the other hand, *Lb. fermentum* P43-01 could inhibit 2 strains of *H. pylori*: strain MS83 and BK364, with high antagonistic activity; therefore, the strain P43-01 was selected for determination of acid and bile tolerance. Survival of *Lb. fermentum* P43-01 after exposures to low pH (2.0 and 3.0) and bile salts (0.3% and 0.5%) was shown in Table 4.7. Decreases in viable cells were observed through the incubation period at pH 2.0. The survival of a selected strain was decreased about 2 log cycles after incubation at pH 2.0 for 3 h. At pH 3.0, the viability of a selected strain was constant at 7.6 $\log_{10}$ cfu/ml during 3 h of incubation. A strain was able to grow in the presence of 0.3 and 0.5% of oxgall. There were increases in viable

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Table 4.7 Acid and bile tolerances of *Lb. fermentum* P43-01

Incubation time (h)	Viable counts (log_{10} cfu/ml)[a]		Incubation time (h)	Viable counts (log_{10} cfu/ml)	
	pH 2.0	pH 3.0		0.3% oxgall	0.5% oxgall
0	7.5 ± 0.2	7.6 ± 0.0	0	7.6 ± 0.1	7.7 ± 0.0
1.5	6.2 ± 0.0	7.6 ± 0.0	3	8.0 ± 0.1	7.8 ± 0.1
3	5.7 ± 0.1	7.6 ± 0.0	6	9.1 ± 0.1	8.7 ± 0.1

[a]Results indicate mean ± SD of two independent experiments

cell count around 1.5 and 1 log cycles after exposures to 0.3 and 0.5% of oxgall for 6 h. Prior to passing through the intestinal tract, probiotics must survive under severe conditions in the stomach with very low pH. Moreover, they have to resist bile acids when they reach the intestine (Dunne *et al.*, 2001). According to acid and bile tolerance, the viable cells of selected strain were substantially declined during incubation at pH 2.0, but the survived cells were remained at about 5.7 log cycles. A strain showed the resistance when it was exposed to pH 3.0. In addition, it could grow in the medium supplemented with 0.3 and 0.5% of bile salts. In agreement with the study of Archer and Halami, most of *Lb. fermentum* strains exhibited high tolerance to low pH as well as high bile concentration with minimum cell count loss (Archer and Halami, 2015). These results revealed that *Lb. fermentum* P43-01 displayed an ability to tolerate an acidic conditions and bile salts, thus, it could be a potential probiotic candidate.

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4.5 Characterization and partial purification of antimicrobial compound

The nature of compounds with anti-*H. pylori* activity of selected LAB strains including *Ln. pseudomesenteroides* FM-S16, *Lb. paracasei* subsp. *tolerans* HB3-4 and *Lb. fermentum* P43-01 were determined. CFSs collected from LAB was neutralized and treated with different enzymes including catalase, pepsin, trypsin, α-chymotrypsin and proteinase K at final concentration of 1 mg/ml and incubated for 3 h at optimal pH and temperature for each specific enzyme. Effect of neutralization, catalase, and protease treatment on inhibitory activity of CFSs obtained from four selected LAB strains was presented as Table 4.8. The inhibitory activity of untreated CFSs from all selected strains were 200 (FM-S16 and P43-01) and 100 (HB3-4) AU/ml, and after CFSs neutralization, their antimicrobial activity did not change. In addition, the inhibitory activity of CFSs against clinical *H. pylori* strains did not alter when treated with catalase. The results indicated that antimicrobial activity did not come from organic acids and H_2O_2. The antimicrobial activity of CFS collected from *Ln. pseudomesenteroides* FM-S16 was absolutely destroyed when it was incubated with proteinase K. The CFS collected from *Lb. fermentum* P43-01 was completely inactivated after incubation with α-chymotrypsin and reduced from 200 to 100 AU/ml when treated with pepsin. These results indicated that the inhibitory substance might be the proteinaceous compounds or bacteriocins. However, anti-*H. pylori* activity of CFS collected from strain HB3-4 still unchanged when exposed to proteases. Anti-*H. pylori* activity from LAB has

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Table 4.8 Inhibitory activity against *H. pylori* of CFS collected from selected strains after neutralization and enzyme treatments

Strain[a]	Target *H. pylori* strain	Anti-*H. pylori* activity (AU/ml)						
		Untreated	Neutralized	Catalase	Pepsin	Trypsin	α-chymotrypsin	Proteinase K
FM-S16	BK364	200	200	200	200	200	200	-
HB3-4	MS83	100	100	100	100	100	100	100
P43-01	MS 83	200	200	200	100	200	-	200
	BK364	200	200	200	100	200	-	200

-; no inhibition.

[a]FM-S16, *Ln. pseudomesenteroides*; HB3-4, *Lb. paracasei* subsp. *tolerans*; P43-01, *Lb. fermentum*.

been reported in many investigations. In addition to lactic acid and low pH, bacteriocin-like substances produced by LAB seem to be an important factor which exerts the inhibition of *H. pylori* growth. *E. faecium* TM39 produced bacteriocin-like substance with antagonistic activity against *H. pylori*, in addition to lactic acid and pH (Tsai *et al.*, 2004). Bacteriocin secreted by *W. confusa* PL9001 was the major substance which suppressed the growth of *H. pylori* (Nam *et al.*, 2002). Six strains of *Bifidobacterium* from human feces showed inhibitory activity against clinical *H. pylori* strains by producing heat-stable proteinaceous compound (Collado *et al.*, 2005). Bulgaricin BB18 secreted by *Lb. bulgaricus* BB18 showed the inhibition against *H. pylori* (Simova *et al.*, 2009). *Lb. brevis* BK11 and *E. faecalis* BK61 produced bacteriocin which displayed strong inhibition on the growth of *H. pylori* (Lim, 2015). Among bacteriocins tested (nisin A, lacticins A164, BH5, JW3, and NK24, pediocin PO2, and leucocin K), lacticins

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A164 and BH5 produced by *Lc. lactis* A164 and *Lc. Lactis* BH5 showed the strongest inhibition on the growth of *H. pylori* (Kim *et al.*, 2003). The effects of AMPs on the growth of *H. pylori* have been less infrequently reported when compared with those of other pathogenic bacteria such as *L. monocytogenes*, *S. aureus*, *C. difficile*, *E. coli* and *Salmonella* spp. More researches that involved AMPs with anti-*H. pylori* activity which produced by LAB were thus needed. Nevertheless, all proteases were not effective on the anti-*H. pylori* activity of CFS collected from *Lb. paracasei* subsp. *tolerans* HB3-4. This is the first report regarding the AMPs produced by lactic acid bacteria isolated from fermented food products in Thailand which showed the inhibition against *H. pylori*.

According to anti- *H. pylori* activity as shown in Table 4. 6, *Ln. pseudomesenteroides* strain FM-S16 exhibited the highest inhibitory activity (12.8 mm of clear zone) on the growth of *H. pylori* BK364. After protease treatment, the antimicrobial activity of CFS was destroyed when exposed to proteinase K. Antimicrobial compounds secreted from *Ln. pseudomesenteroides* FM-S16 might be bacteriocins. Therefore, partial purification and some characteristics of bacteriocin FM-S16 were evaluated.

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The CFS from *Ln. pseudomesenteroides* FM-S16 was harvested from 18-h-old culture in MRS medium. The bacteriocin FM-S16 was purified by a three-step procedure including hydrophobic interaction, cation- exchange chromatography and reverse-phase HPLC. A summary of purification process was shown in Table 4.9 Forty percent of the activity was recovered and the purity increased up to 2.7 folds after using hydrophobic interaction chromatography (Amberlite XAD-16). Then, the proteinaceous solution was passed through cation- exchange column (SP- sepharose) and the bacteriocin activity was recovered in unbound fraction. This step increased purity 16.8 folds and gave a slightly decrease in the recovery (38%). One milliliters of active fraction was subjected to reverse-phase HPLC. Five peaks showing the absorbance at wavelength 220 nm were collected and test their antimicrobial activity on *H. pylori* growth (Figure 4.11). Two fractions (4-1 and 4-2) corresponding to peak no.4 expressed 200 and 400 of their anti-*H. pylori* activity (Table 4.10). Finally, 0.1% of the total activity yield and 380 purity fold of culture supernatant were obtained when theses purification steps were carried out. Fraction 4-2 containing the highest of antimicrobial activity was analyzed by MALDI-TOF/TOF mass spectrometry. The mass of bacteriocin FM-S16 was in the range of 1094-1316 Da as shown in Figure 4.12.

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Table 4.9 Partial purification of bacteriocin FM-S16 produced by *Ln. pseudomesenteroides* FM-S16

Purification step	Volume (mL)	Total activity[a]	Yield (%)	Total protein[b]	Specific activity	Purity (fold)
Supernatant	1000	400,000	100	16000	25.0	1
Amberlite	200	160,000	40	2400	66.7	2.7
SP-sepharose	94	150,000	38	358	419	16.8
RP-HPLC	1	400	0.1	0.042	9500	380

[a]Antimicrobial activity [in arbitrary units (AU)] was assayed by the spot-on-lawn method using *H. pylori* BK364 as an indicator strain. [b]The protein concentration (in mg/ml) was determined by the bicinchoninic acid assay (Smith *et al*., 1985)

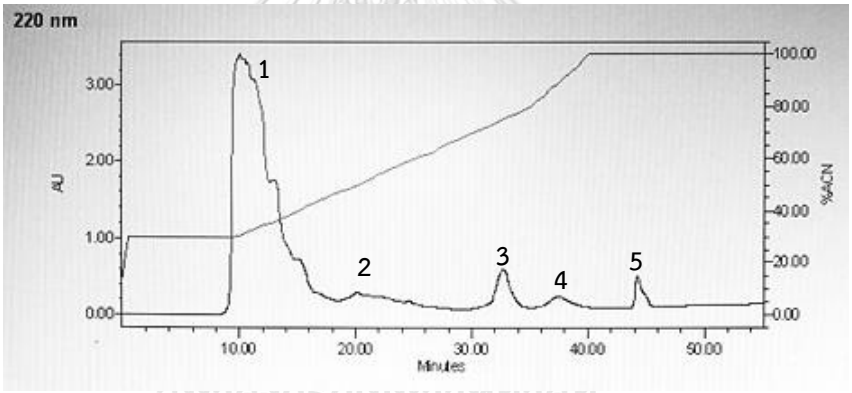

Figure 4.11 The separation of bacteriocins from *Ln. pseudomesenteroides* FM-S16 with reversed phase-HPLC. The bacteriocin activity was detected at wavelenght 220 nm in the fraction containing in the peaks no.4.

Table 4.10 Anti-*H. pylori* activity of fractions in the peak no.4

Fraction	Anti *H. pylori* activity (AU/ml)
4-1	200
4-2	400

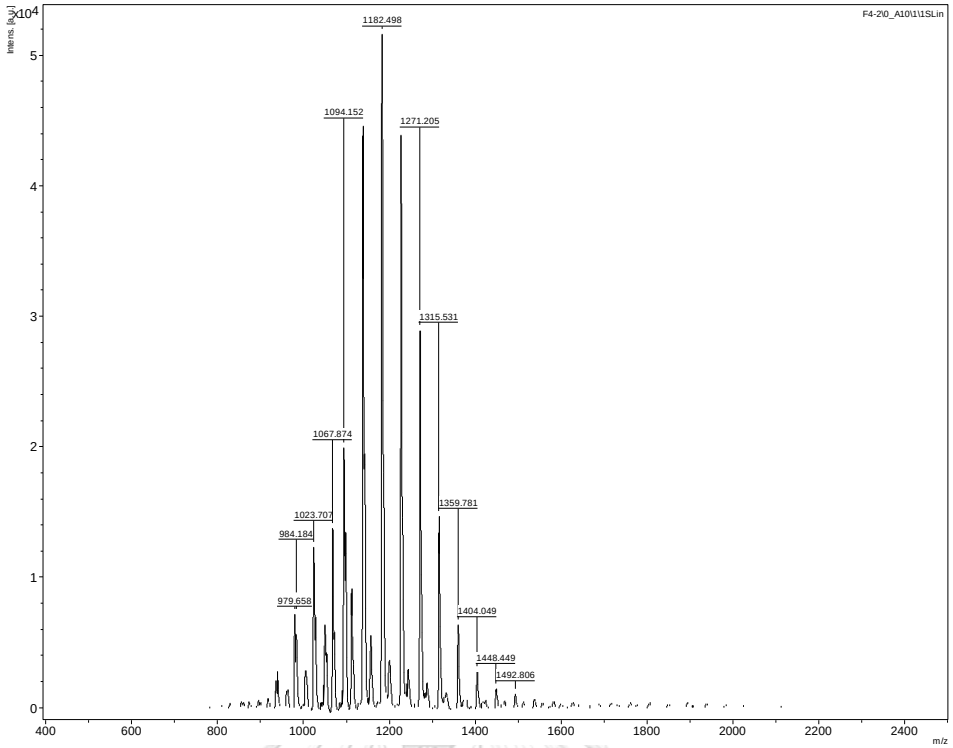

Figure 4.12 The MALDI-TOF/TOF mass spectra of the fraction containing in the fraction 4-2

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CHAPTER 5

CONCLUSION

During the investigation, a total of 238 of LAB strains isolated from plant materials and fermented food products including West-Indian Jasmine, coffee cherries, mushroom, fermented tea leaves, fermented plant beverage, fermented mushroom and fermented rice noodle collected in Thailand were screened for antibacterial activity. Fifty-three strains displayed the different spectrum of antibacterial activity against three clinical strains of *H. pylori*. On the basis of the phenotypic characteristics and 16S rRNA gene sequence analyses, the strains were belonged to the genus *Lactobacillus* (20 strains), *Pediococcus* (2 strains), *Enterococcus* (3 strains), *Leuconostoc* (6 strains), *Lactococcus* (4 strains) and *Weissella* (18 strains). They were divided into 16 groups which were identified as *Lactobacillus plantarum* subsp. *plantarum* (3 strains, Group 1), *Lactobacillus pentosus* (10 starins, Group 2), *Pediococcus pentosaceus* (2 strains, Group 3), *Lactobacillus paracasei* subsp. *tolerans* (1 strain, Group 4), *Lactobacillus fermentum* (5 strains, Group 5), *Enterococcus faecium* (2 strains, Group 6), *Enterococcus casseliflavus* (1 strain, Group 7), *Lactococcus garvieae* subsp. *garvieae* (1 strain, Group 8), *Lactococcus lactis* subsp. *lactis* (3 strains, Group 9), *Leuconostoc lactis* (2 strains, Group 10), *Leuconostoc citreum* (2 strains, , Group 11), *Leuconostoc pseudomesenteroides* (2 strains, , Group 12), *Weissella paramesenteroides* (1 strain, Group 13), *Weissella cibaria* (6 strains, Group 14) and

Weissella confusa (11 strains, Group 15), respectively. *Lb. pentosus*, *P. pentosaceus*, *Lc. lactis* subsp. *lactis*, *Ln. lactis* and *W. cibaria* were isolated from mushroom. *En. casseliflavus, Lc. lactis subsp. lactis* and *W. cibaria* were found in Miang. *Lb. plantarum* subsp. *plantarum*, *Lc. garvieae* subsp. *garvieae*, *Ln. pseudomesenteroides*, *W. paramesenteroides* and *W. confusa* were distributed in fermented plant beverage. *Lb. plantarum* subsp. *plantarum*, *Ln. pseudomesenteroides*, *W. cibaria* and *W. confusa* were isolated from fermented mushroom. *Lb. pentosus*, *Lb. paracasei* subsp. *tolerans*, *Lb. fermentum*, *En. faecium*, *Lc. lactis* subsp. *lactis*, *Ln. lactis*, *Ln. citreum* and *W. confusa* were found in Khanom-jeen. A novel species in group 16, *Lactobacillus* sp. Ru20-1 isolated from West-Indian jasmine (*Ixora coccinea* L.), was proposed as *Lactobacillus ixorae*, according to the phenotypic, chemotaxonomic and genotypic characteristics.

Nine strains were selected to determine their adherence abilities onto AGS cell lines. *Ln. pseudomesenteroides* strain BGM-S4 and FM-S16 expressed the adherence, 20.4% and 12.9%, respectively. The remained strains exhibited the adherence levels less than 5%. *Lb. fermentum* P43-01 could resist to acid conditions at pH 3. Moreover, this strain could tolerate to bile acid and propagate their number during the incubation period.

Cell-free culture supernatants (CFSs) collected from strain FM-S16, HB3-4 and P43-01 were selected to treat with proteases. After treatment, CFSs from isolates FM-S16 and P43-01 had no activity against *H. pylori*. The result revealed that the

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antimicrobial compounds in the CFSs of the two strains might be bacteriocins. Therefore, the compounds in the CFS of strain FM-S16 that exhibited the highest anti-*H. pylori* activity was selected for bacteriocin purification. After performing a three-step procedure including hydrophobic interaction, cation-exchange chromatography and reverse-phase HPLC, 0.1% of yield and 380 folds of purity of CFS were obtained. The molecular mass of bacteriocin in an active fraction was in the range of 1094-1316 Da.

The results from this study provided the distribution of LAB in plant materials and fermented food products collected in Thailand which are the good isolation sources of lactic acid bacteria. According to the results of screening the probiotic properties, these strains can be considered as the good candidates for use as probiotics for management of *H. pylori* infections in patient. Nevertheless, further investigations in an *in vivo* model are required to evaluate the information.

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APPENDIX A

Culture media

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

1. MRS agar

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

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

2. Columbia blood agar base supplemented with 7% sheep blood

Pancreatic Digest of Casein	10.0	g

Proteose Peptone No. 3	5.0	g
Yeast Extract	5.0	g
Beef Heart, Infusion from 500 g.	3.0	g
Corn Starch	1.0	g
Sodium Chloride	5.0	g
Agar	15.0	g
Distilled water	1.0	L

After autoclaving, placed the media in water bath with the temperature of 55 ºC, then added with 70 ml of sheep blood and gentle mixed before pouring to the plates.

3. Brain heart infusion agar

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

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

4. Salt solution

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

5. L-(+) Arginine agar medium

Peptone	1.0	g
K_2HPO_4	0.3	g
NaCl	5	g
L-(+) Arginine hydrochloride	10	g
Phenol red	0.01	g
Agar	3.0	g
Distilled water	1	l

Adjust to pH 6.8

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6. Nitrate broth

Yeast extract	5	g
Peptone	10	g
KNO_3	1	g
NaCl	10	g
Distilled water	1	L

Adjust pH to 6.8

APPENDIX B

Reagents and buffers for bacterial identification

1. 0.85% NaCl

NaCl	0.85	g
Distilled water	100	ml

2. 1X Phosphate buffer saline (PBS buffer)

Yeast extract	5	g
Peptone	10	g
KNO_3	1	g
NaCl	10	g
Distilled water	1	L
Adjust pH to 6.8		

3. Nitrate reduction test reagent

Sulphanilic acid solution

Sulphanilic acid	0.8	g
5 N Acetic acid	100	ml

N,N-dimethyl-1-naphthylamine solution

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

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The ability to reduce nitrate to nitrite was determined using nitrate broth. After incubation for 7 days, the culture broth was added with nitrate reduction test reagent. The pink to red color represented the presence of nitrite (positive).

4. 3% H_2O_2 for catalase test

H_2O_2	3	ml
Distilled water	97	ml

Catalase test was examined by transferring one colony of pure culture onto the slide and then placed 3% of hydrogen peroxide onto the slide. The formation of gas bubbles indicating a positive test was observed.

5. Mix indicator

Bromthymol blue	0.2	g
Neutral red	0.1	g
Ethanol	300	ml

6. PCR reaction mixture

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Stock		**1 volume (100 µl)**
Forward Primer: 20F	10 pmol/µl	4
Reverse Primer: 1492R	10 pmol/µl	4
10 x Taq buffer (NH_4SO_4-$MgCl_2$)	10 x	10
dNTP	2.0 mM	2
$MgCl_2$	25 mM	8
Taq DNA polymerase	5 Unit/µl	0.5
Milli-Q water	-	66.5

Template	Undilute	5

7. 1X Tris-acetate (TAE) buffer

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

8. 0.8% Agarose gel

Agarose	0.8	g
Distilled water	100	ml

Melt the mixture in microwave until obtaining the homogeneous solution for casting a gel.

9. Ethidium bromide solution (10 mg/ml)

Ethidium bromide	1	g
Distilled water	100	ml

10 . Cellular fatty acid analysis

10.1 Reagent 1 Saponification reagent

NaOH	15	g
MeOH (HPLC grade)	50	ml
Milli-Q water	50	ml

Dissolve NaOH in Milli-Q water and add MeOH

10.2 Reagent 2 Methylation reagent

6 N HCl	65	ml
MeOH (HPLC grade)	55	ml

pH of the solution must be below 1.5.

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10.3 Reagent 3 Extraction solvent

n-Hexane (HPLC grade)	50	ml
Methyl-tert-Butyl ether	50	ml

10.4 Reagent 4 base wash reagent

Sodium hydroxide	1.2	ml
Milli-Q water	100	ml

10.5 Reagent 5 Saturated sodium chloride

Sodium chloride saturated in Milli-Q water

11. RNase A solution

RNase A	20	ng
0.15 M NaCl, pH 5.0	10	ml

Dissolve RNase A in 0.15 M NaCl, pH 5.0 and heat at 95 ºC for 5-10 min. Keep RNase A solution at -20 ºC.

12. RNase T solution

RNase T	800	U
0.1 M Tris-HCl (pH 7.2)	1	ml

Mix RNase T in 0.1M Tris-HCl (pH 7.2) and heat at 95ºC for 5 min. Keep RNase T solution at -20 ºC.

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APPENDIX C

Buffers and reagents for partial purification of bacteriocin

1. 40% Ethanol

Absolute ethanol	40	ml
Milli-Q water	60	ml

2. 70% iso-propanol + 0.1% Trifluoroacetic acid (TFA)

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

3. 50 mM Sodium phosphate buffer (pH 7.2)

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

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

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4. 50 mM Sodium phosphate buffer (pH 7.2) + 0.25 M, 0.5 M, 0.75 or 1 M NaCl

Prepare with the same ingredients with 50 mM sodium phosphate buffer and add NaCl of 14.62 g, 29.25 g, 43.88 g and 58.5 g of NaCl to get the buffers containing 0.25 M, 0.5 M, 0.75 and 1 M of NaCl, respectively.

5. BCA protein determination (Pierce™ BCA Protein Assay Kit, Thermo Sciencetific)

5.1 Preparation of diluted albumin (BSA) standards

Dilute the contents of one albumin standard (BSA) ampule into several clean vials, preferably using the same diluent as the sample(s). Each 1ml ampule of 2 mg/ml albumin standard is sufficient to prepare a set of diluted standards for either working range suggested in Table 1.

Table 1 Preparation of diluted albumin (BSA) standards

Vial	Volume of diluent (µl)	Volume and source of BSA (µl)	Final concentration of BSA (µg/ml)
A	0	300 of Stock	2000
B	125	325 of Stock	1500
C	325	375 of Stock	1000
D	175	175 of vial B dilution	750
E	325	325 of vial C dilution	500
F	325	325 of vial E dilution	250
G	325	325 of vial F dilution	125
H	400	100 of vial G dilution	25
I	400	0	0 = Blank

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5.2 Preparation of the BCA working reagent (WR)

Prepare WR by mixing 50 parts of BCA Reagent A with 1 part of BCA Reagent B (50:1, Reagent A:B).

5.3 Microplate Procedure (Sample to WR ratio = 1:8)

1) Pipette 25μl of each standard or unknown sample replicate into a microplate well (working range = 20-2000 μg/ml).

Note: If sample size is limited, 10 μl of each unknown sample and standard can be used (sample to WR ratio = 1:20). However, the working range of the assay in this case will be limited to 125-2000 μg/mL.

2) Add 200 μl of the WR to each well and mix plate thoroughly on a plate shaker for 30 seconds.

3) Cover plate and incubate at 37°C for 30 minutes.

4) Cool plate to RT. Measure the absorbance at or near 562 nm on a plate reader.

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APPENDIX D

Scientific name of plant samples and 16S rRNA and *pheS* gene sequences

1) Scientific name of plants samples used in this study

Sample	Source	Collection period	Scientific name
a. West-Indian jasmine	Kalasin	January to Febuary 2015	*Ixora coccinea* L.
b. Coffee cherries	Chiang Mai	May – June 2013	*Coffea arabica* L.
c. Mushroom	Phare	May – June 2013	*Russula emetica* (Schaeff.) Pers
	Lamphun	May – June 2013	*Tricholoma crissum* (Berk.) Sacc. *Boletus colossus* Heim. *Geastrum saccatum* Fr.
	Lampang	May – June 2013	*Russula emetica* (Schaeff.) Pers *Geastrum saccatum* Fr. *Lentinus polychrous* Lev. *Russula virescens* (schaeff.) fr. *Russula foetens* Fr. *Amanita vaginata*
d. Fermented tea leaves (*Miang*)	Chiang Mai	August – September 2013	*Camellia sinensis*
	Lampang	August – September 2013	*Camellia sinensis*
e. Fermented mushroom	Nakhonratchasima	August – September 2013	*Pleurotus pulmonarius*

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2) 16S rRNA gene sequences

1) Strain BGM-S6 (1,332 bp)

TAACACGTGGGAAACCTGCCCAGAAGCGGGGGATAACACCTGGAAACAGATGCTAATACCGCATAACAACTTG

GACCGCATGGTCCGAGCTTGAAAGATGGCTTCGGCTATCACTTTTGGATGGTCCCGCGGCGTATTAGCTAGAT

GGTGGGGTAACGGCTCACCATGGCAATGATACGTAGCCGACCTGAGAGGGTAATCGGCCACATTGGGACTGA

GACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGAAAGTCTGATGGAGCAAC

GCCGCGTGAGTGAAGAAGGGTTTCGGCTCGTAAAACTCTGTTGTTAAAGAAGAACATATCTGAGAGTAACTGT

TCAGGTATTGACGGTATTTAACCAGAAAGCCACGGCTCAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTG

GCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTTTTAAGTCTGATGTGAAAGCCTTCG

GCTCAACCGAAGAAGTGCATCGGAAACTGGGAAACTTGAGTGCAGAAGAGGACAGTGGAACTCCATGTGTAGC

GGTGAAATGCGTAGATATATGGAAGAACACCAGTGGCGAAGGCGGCTGTCTGGTCTGTAACTGACGCTGAGGC

TCGAAAGTATGGGTAGCAAACAGGATTAGATACCCTGGTAGTCCATACCGTAAACGATGAATGCTAAGTGTTG

GAAGGGTTTCCGCCCTTCAATGCTGCAGCTAACGCATTAAGCATTCCCCCCGGGGGAATACGGCCCCCAAGGC

TGAAACTCAAAGGAATTTGACGGGGGCCCGCACAAGCGGTGGAACATGTGGTTTAATTCGAAGCTACGCGAAG

AACCTTACCAGGTCTTGACATACTATGCAAATCTAAGAGATTAGACGTTCCCTTCGGGGACATGGATACAGGT

GGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATTATC

AGTTGCCAGCATTAAGTTGGGCACTCTGGTGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCA

AATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGATGGTACAACGAGTTGCGAACTCGCGA

GAGTAAGCTAATCTCTTAAAGCCATTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGTCGGAATC

GCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATG

AGAGTTTGTAACACCCAAA

2) Strain SB1-2 (1,399 bp)

CTCTGGTATTGATTGGTGCTTGCATCATGATTTACATTTGAGTGAGTGGCGAACTGGTGAGTAACACGTGGGA
AACCTGCCCAGAAGCGGGGGATAACACCTGGAAACAGATGCTAATACCGCATAACAACTTGGACCGCATGGTC
CGAGTTTGAAAGATGGCTTCGGCTATCACTTTTGGATGGTCCCGCGGCGTATTAGCTAGATGGTGAGGTAACG
GCTCACCATGGCAATGATACGTAGCCGACCTGAGAGGGTAATCGGCCACATTGGGACTGAGACACGGCCCAAA
CTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGAAAGTCTGATGGAGCAACGCCGCGTGAGTGA
AGAAGGGTTTCGGCTCGTAAAACTCTGTTGTTAAAGAAGAACATATCTGAGAGTAACTGTTCAGGTATTGACG
GTATTTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCG
GATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTTTTAAGTCTGATGTGAAAGCCTTCGGCTCAACCGAAGAA
GTGCATCGGAAACTGGGAAACTTGAGTGCAGAAGAGGACAGTGGAACTCCATGTGTAGCGGTGAAATGCGTAG
ATATATGGAAGAACACCAGTGGCGAAGGCGGCTGTCTGGTCTGTAACTGACGCTGAGGCTCGAAAGTATGGGT
AGCAAACAGGATTAGATACCCTGGTAGTCCATACCGTAAACGATGAATGCTAAGTGTTGGAGGGTTTCCGCCC
TTCAGTGCTGCAGCTAACGCATTAAGCATTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATT
GACGGGGGCCCGCACAAGCGGTGGAGCATGGGTTTAATTCGAAGCTACGCGAAGAACCTTACCAGGTCTTGAC
ATACTATGCAAATCTAGAGATTAGACGTTCCCTTCGGGGACATGGATACAGGTGGTGCATGGTTGTCGTCAGC
TCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATTATCAGTTGCCAGCATTAAGTTGG
GCACTCTGGTGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGA
CCTGGGCTACACACGTGCTACAATGGATGGTACAACGAGTTGCGAACTCGCGAGAGTAAGCTAATCTCTTAAA
GCCATTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGTCGGAATCGCTAGTAATCGCGGATCAGC
ATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGAGAGTTTGTAACACCCAAAG
TCGGTGGGGTAA

3) Strain MRA3-4 (1,393 bp)

TGATTATGACGTACTTGTACTGATTGAGATTTTAACACGAAGTGAGTGGCGAACGGGTGAGTAACACGTGGGT
AACCTGCCCAGAAGTAGGGGATAACACCTGGAAACAGATGCTAATACCGTATAACAGAGAAAACCGCATGGTT

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TTCTTTTAAAAGATGGCTCTGCTATCACTTCTGGATGGACCCGCGGCGTATTAGCTAGTTGGTGAGGTAAAGG

CTCACCAAGGCAGTGATACGTAGCCGACCTGAGAGGGTAATCGGCCACATTGGGACTGAGACACGGCCCAGAC

TCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGCAAGTCTGATGGAGCAACGCCGCGTGAGTGAA

GAAGGGTTTCGGCTCGTAAAGCTCTGTTGTTAAAGAAGAACGTGGGTAAGAGTAACTGTTTACCCAGTGACGG

TATTTAACCAGAAAGCCACGGCTAATACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGA

TTTATTGGGCGTAAAGCGAGCGCAGGCGGTCTTTTAAGTCTAATGTGAAAGCCTTCGGCTCAACCGAAGAAGT

GCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGACAGTGGAACTCCATGTGTAGCGGTGAAATGCGTAGAT

ATATGGAAGAACACCAGTGGCGAAGGCGGCTGTCTGGTCTGCAACTGACGCTGAGGCTCGAAAGCATGGGTAG

CGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGATGATTACTAAGTGTTGGAGGGTTTCCGCCCTT

CAGTGCTGCAGCTAACGCATTAAGTAATCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAAGAATTGA

CGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCTACGCGAAGAACCTTACCAGGTCTTGACA

TCTTCTGACAGTCTAAGAGATTAGAGGTTCCCTTCGGGGACAGAATGACAGGTGGTGCATGGTTGTCGTCAGC

TCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATTACTAGTTGCCAGCATTAAGTTGG

GCACTCTAGTGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGACGACGTCAAATCATCATGCCCCTTATGA

CCTGGGCTACACACGTGCTACAATGGATGGTACAACGAGTCGCGAGACCGCGAGGTTAAGCTAATCTCTTAAA

ACCATTCTCAGTTCGGACTGTAGGCTGCAACTCGCCTACACGAAGTCGGAATCGCTAGTAATCGCGGATCAGC

ATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGAGAGTTTGTAACACCCAAAG

CCGGTG

4) Strain HB3-4 (1,403 bp)

GAGTTCTCGTTGATGATCGGTGCTTGCACCGAGATTCAACATGGAACGAGTGGCGGACGGGTGAGTAACACGT

GGGTAACCTGCCCTTAAGTGGGGGATAACATTTGGAAACAGATGCTAATACCGCATAGATCCAAGAACCGCAT

GGTTCTTGGCTGAAAGATGGCGTAAGCTATCGCTTTTGGATGGACCCGCGGCGTATTAGCTAGTTGGTGAGGT

AATGGCTCACCAAGGCGATGATACGTAGCCGAACTGAGAGGTTGATCGGCCACATTGGGACTGAGACACGGCC

CAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGCAAGTCTGATGGAGCAACGCCGCGTGA

GTGAAGAAGGCTTTCGGGTCGTAAAACTCTGTTGTTGGAGAAGAATGGTCGGCAGAGTAACTGTTGTCGGCGT

GACGGTATCCAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTA

TCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTTTTAAGTCTGATGTGAAAGCCCTCGGCTTAACCGA

GGAAGCGCATCGGAAACTGGGAAACTTGAGTGCAGAAGAGGACAGTGGAACTCCATGTGTAGCGGTGAAATG

CGTAGATATATGGAAGAACACCAGTGGCGAAGGCGGCTGTCTGGTCTGTAACTGACGCTGAGGCTCGAAAGCA

TGGGTAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGATGAATGCTAGGTGTTGGAGGGTTTC

CGCCCTTCAGTGCCGCAGCTAACGCATTAAGCATTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAG

GAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGT

CTTGACATCTTTTGATCACCTGAGAGATCAGGTTTCCCCTTCGGGGGCAAAATGACAGGTGGTGCATGGTTGT

CGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATGACTAGTTGCCAGCATT

TAGTTGGGCACTCTAGTAAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCC

CTTATGACCTGGGCTACACACGTGCTACAATGGATGGTACAACGAGTTGCGAGACCGCGAGGTCAAGCTAATC

TCTTAAAGCCATTCTCAGTTCGGACTGTAGGCTGCAACTCGCCTACACGAAGTCGGAATCGCTAGTAATCGCG

GATCAGCACGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGAGAGTTTGTAACA

CCCGAAGCCGGTGGCGT

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5) Strain P43-01 (1,395 bp)

TGATTGATGGTGCTTGCACCTGATTGATTTTGGTCGCCAACGAGTGGCGGACGGGTGAGTAACACGTAGGTAA

CCTGCCCAGAAGCGGGGGACAACATTTGGAAACAGATGCTAATACCGCATAACAACGTTGTTCGCATGAACAA

CGCTTAAAAGATGGCTTCTCGCTATCACTTCTGGATGGACCTGCGGTGCATTAGCTTGTTGGTGGGGTAATGG

CCTACCAAGGCGATGATGCATAGCCGAGTTGAGAGACTGATCGGCCACAATGGGACTGAGACACGGCCCATAC

TCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGGCGCAAGCCTGATGGAGCAACACCGCGTGAGTGA

AGAAGGGTTTCGGCTCGTAAAGCTCTGTTGTTAAAGAAGAACACGTATGAGAGTACTGTTCATACGTTGACGG

TATTAACCAGAAAGTCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGA

TTTATTGGGCGTAAAGAGAGTGCAGGCGGTTTTCTAAGTCTGATGTGAAAGCCTTCGGCTTAACCGGAGAAGT

GCATCGGAAACTGGATAACTTGAGTGCAGAAGAGGGTAGTGGAACTCCATGTGTAGCGGTGGAATGCGTAGAT

ATATGGAAGAACACCAGTGGCGAAGGCGGCTACCTGGTCTGCAACTGACGCTGAGACTCGAAAGCATGGGTAG

CGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGATGAGTGCTAGGTGTTGGAGGGTTTCCGCCCTT

CAGTGCCGGAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGA

CGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCTACGCGAAGAACCTTACCAGGTCTTGACA

TCTTGCGCCAACCCTAGAGATAGGGCGTTTCCTTCGGGAACGCAATGACAGGTGGTGCATGGTCGTCGTCAGC

TCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTTACTAGTTGCCAGCATTAAGTTGG

GCACTCTAGTGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGACGACGTCAGATCATCATGCCCCTTATGA

CCTGGGCTACACACGTGCTACAATGGACGGTACAACGAGTCGCGAACTCGCGAGGGCAAGCAAATCTCTTAAA

ACCGTTCTCAGTTCGGACTGCAGGCTGCAACTCGCCTGCACGAAGTCGGAATCGCTAGTAATCGCGGATCAGC

ATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGAGAGTTTGTAACACCCAAAG

TCGGTGGGG

6) Strain N21-9 (1,373 bp)

GCTCCACCGGAAAAAGAGGAGTGGCGAACGGGTGAGTAACACGTGGGTAACCTGCCCATCAGAAGGGGATAA

CACTTGGAAACAGGTGCTAATACCGTATAACAATCGAAACCGCATGGTTTTGATTTGAAAGGCGCTTTCGGGT

GTCGCTGATGGATGGACCCGCGGTGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCCACGATGCATAG

CCGACCTGAGAGGGTGATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGG

GAATCTTCGGCAATGGACGAAAGTCTGACCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAAC

TCTGTTGTTAGAGAAGAACAAGGATGAGAGTAACTGTTCATCCCTTGACGGTATCTAACCAGAAAGCCACGGC

TAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGC

GCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGAGACTT

GAGTGCAGAAGAGGAGAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAGGAACACCAGTGGC

GAAGGCGGCTCTCTGGTCTGTAACTGACGCTGAGGCTCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTG

GTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTGGAGGGTTTCCGCCCTTCAGTGCTGCAGCTAACGCATT

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AAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGT

GGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTTTGACCACTCTAGAGA

TAGAGCTTCCCCTTCGGGGGCAAAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGG

TTAAGTCCCGCAACGAGCGCAACCCTTATTGTTAGTTGCCATCATTCAGTTGGGCACTCTAGCAAGACTGCCG

GTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTA

CAATGGGAAGTACAACGAGTTGCGAAGTCGCGAGGCTAAGCTAATCTCTTAAAGCTTCTCTCAGTTCGGATTG

CAGGCTGCAACTCGCCTGCATGAAGCCGGAATCGCTAGTAATCGCGGATCAGCACGCCGCGGTGAATACGTTC

CCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGAGG

7) Strain FTLE-5 (1,376 bp)

CCGGAGCTTGCTCCACCGAAAGAAAAAGAGTGGCGAACGGGTGAGTAACACGTGGGTAACCTGCCCATCAGAA

GGGGATAACACTTGGAAACAGGTGCTAATACCGTATAACACTATTTTCCGCATGGAAGAAAGTTGAAAGGCGC

TTTTGCGTCACTGATGGATGGACCCGCGGTGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGAT

GCATAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGC

AGTAGGGAATCTTCGGCAATGGACGAAAGTCTGACCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCG

TAAAACTCTGTTGTTAGAGAAGAACAAGGATGAGAGTAAAACGTTCATCCCTTGACGGTATCTAACCAGAAAG

CCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAA

AGCGAGCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGG

GAGACTTGAGTGCAGAAGAGGAGAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAGGAACAC

CAGTGGCGAAGGCGGCTCTCTGGTCTGTAACTGACGCTGAGGCTCGAAAGCGTGGGGAGCGAACAGGATTAG

ATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTGGAGGGTTTCCGCCCTTCAGTGCTGCAGCA

AACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCAC

AAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTTTGACCACT

CTAGAGATAGAGCTTCCCCTTCGGGGGCAAAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGA

TGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATTGTAGTTGCCACATTTAGTTGGGCACTCTAGCGAGAC

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TGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACG

TGCTACAATGGGAAGTACAACGAGTTGCGAAGTCGCGAGGCTAAGCTAATCTCTTAAAGCTTCTCTCAGTTCG

GATTGTAGGCTGCAACTCGCCTACATGAAGCCGGAATCGCTAGTAATCGCGGATCAGCACGCCGCGGTGAATA

CGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGATAGTCGGT

8) Strain BGM-S2 (1,339 bp)

ATTTTTATGAAGAGCGGCGAACGGGTGAGTAACGCGTGGGAAATCTGCCGAGTAGCGGGGGACAACGTTTGGA

AACGAACGCTAATACCGCATAACAATGAGAATCGCATGATTCTTATTTAAAAGAAGCAATTGCTTCACTACTTG

ATGATCCCGCGTTGTATTAGCTAGTTGGTAGTGTAAAGGACTACCAAGGCGATGATACATAGCCGACCTGAGA

GGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGC

AATGGGGGCAACCCTGACCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATGTAAAACTCTGTTGTTAGA

GAAGAACGTTAAGTAGAGTGGAAAATTACTTAAGTGACGGTATCTAACCAGAAAGGGACGGCTAATACGTGCC

AGCAGCCGCGGTAATACGTAGGTCCCAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGTGGTTTCT

TAAGTCTGATGTAAAAGGCAGTGGCTCAACCATTGTGTGCATTGGAAACTGGGAGACTTGAGTGCAGGAAGGA

GAGTGGAATTCCTGGTAGCGGTGAAATGCGTAGATTATGGAGGAACACCGGAGCGAAAGCGGCTCTCTGGCCT

GTAACTGACACTGAGGCTCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACG

ATGAGTGCTAGCTGTAGGGAGCTATAAGTTCTCTGTAGCGCAGCTAACGCATTAAGCACTCCGCCGGGGAGTA

CGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCAGTGGTTTAATTCGAAG

CAACGCGAAGAACCTTACCAGGTCTTGACATACTCGTGCTATCCTTAGAATAAGGAGTTCCTTCGGGACACGG

GATACAGGTGGTGCATGGTTGTCTCAGCTCTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCT

TATTACTAGTTGCCATCATTAAGTTGGGCACTCTAGTGAGACTGCCGGTGATAAACCGGAGGAAGGTGGGGAT

GACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGATGGTACAACGAGTCGCCAA

CCCGCGAGGGTGCGCTAATCTCTTAAAACCATTCTCAGTTCGGATTGCAGGCTGCAACTCGCCTGCATGAAGT

CGGAATCGCTAGTAATCGCGGATCAGCACGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCA

CACCACGGAAGTTGGGAGTACCCAA

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9) Strain P43-1A (1,359 bp)

TTGTACCGACTGGATGAGCAGCGAACGGGTGAGTAACGCGTGGGGAATCTGCCTTTGAGCGGGGGACAACATT
TGGAAACGAATGCTAATACCGCATAAAAACTTTAAACACAAGTTTTAAGTTTGAAAGATGCAATTGCATCACTC
AGAGATGATCCCGCGTTGTATTAGCTAGTTGGTGAGGTAAAGGCTCACCAAGGCGATGATACATAGCCGACCT
GAGAGGGTGATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTT
CGGCAATGGACGAAAGTCTGACCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTTG
GTAGAGAAGAACGTTGGTGAGAGTGGAAAGCTCATCAAGTGACGGTAACTACCCAGAAAGGGACGGCTAACTA
CGTGCCAGCAGCCGCGGTAATACGTAGGTCCCGAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGT
GGTTTATTAAGTCTGGTGTAAAAGGCAGTGGCTCAACCATTGTATGCATTGGAAACTGGTAGACTTGAGTGCA
GGAGAGGAGAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAGGAACACCGGTGGCGAAAGC
GGCTCTCTGGCCTGTAACTGACACTGAGGCTCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTC
CACGCCGTAAACGATGAGTGCTAGATGTAGGGAGCTATAAGTTCTCTGTATCGCAGCTAACGCAATAAGCACT
CCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCAT
GGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATACTCGTGCTATTCTAGAGATAGGAAGT
TCCTTCGGGACACGGGATACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCC
GCAACGAGCGCAACCCCTATTGTTAGTTGCCATCATTAAGTTGGGCACTCTAACGAGACTGCCGGTGATAAAC
CGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGATG
GTACAACGAGTCGCGAGACAGTGATGTTTAGCTAATCTCTTAAAACCATTCTCAGTTCGGATTGTAGGCTGCA
ACTCGCCTACATGAAGTCGGAATCGCTAGTAATCGCGGATCAGCACGCCGCGGTGAATACGTTCCCGGGCCTT
GTACACACCGCCCGTCACACCACGGGAGTTGGGAGTACCCGAAGTAG

10) Strain P32-9 (1,361 bp)

TTGCACCTTTCAAGCGAGTGGCGAACGGGTGAGTAACACGTGGATAACCTGCCTCAAGGCTGGGGATAACATT
TGGAAACAGATGCTAATACCGAATAAAACTTAGTATCGCATGATACAAAGTTGAAAGGCGCTACGGCGTCACC
TAGAGATGGGTCCGCGGTGCATTAGTTAGTTGGTGGGGTAAAGGCCTACCAAGACAATGATGCATAGCCGAGT

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TGAGAGACTGATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCTGCAGTAGGGAATCTT
CCACAATGGGCGCAAGCCTGATGGAGCAACGCCGCGTGTGTGATGAAGGCTTTCGGGTCGTAAAGCACTGTTG
TATGGGAAGAAATGCTAGAATAGGGAATGATTCTAGTTCGACGGTACCATACCAGAAAGGGACGGCTAAATAC
GTGCCAGCAGCCGCGGTAATACGTATGTCCCGAGCGTTATCCGGATTTATTGGGCGTAAAGCGAGCGCAGACG
GTTGATTAAGTCTGATGTGAAAGCCCGGAGCTCAACTCCGGAATGGCATTGGAAACTGGTTAACTTGAGTGTT
GTAGAGGTAAGTGGAACTCCATGTGTAGCGGTGGAATGCGTAGATATATGGAAGAACACCAGTGGCGAAGGCG
GCTTACTGGACAACAACTGACGTTGAGGCTCGAAAGTGTGGGTAGCAAACAGGATTAGATACCCTGGTAGTCC
ACACCGTAAACGATGAATACTAGGTGTTAGGAGGTTTCCGCCTCTTAGTGCCGAAGCTAACGCATTAAGTATT
CCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGAGCAT
GTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTTTGAAGCTTCTAGAGATAGAAGT
GTTCTCTTCGGAGACAAAGTGACAGGTGGTGCATGGTCGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGT
CCCGCAACGAGCGCAACCCTTATTGTTAGTTGCCAGCATTCAGTTGGGCACTCTAGCGAGACTGCCGGTGACA
AACCGGAGGAAGGCGGGGACGACGTCAGATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGG
CGTATACAACGAGTTGCCAGCCCGCGAGGGTGAGCTAATCTCTTAAAGTACGTCTCAGTTCGGACTGCAGTCT
GCAACTCGACTGCACGAAGTCGGAATCGCTAGTAATCGCGGATCAGCACGCCGCGGTGAATACGTTCCCGGGT
CTTGTACACACCGCCCGTCACACCATGGGAGTTTGTAATGCCCAAAGC

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11) Strain P42-1A (1,350 bp)

GGTGAGTAACACGTGGATAACCTGCCTCAAGGCTGGGGATAACATTTGGAAACAGATGCTAATACCGAATAAA
ACTTAGTATCGCATGATATCAAGTTAAAAGGCGCTACGGCGTCACCTAGAGATGGATCCGCGGTGCATTAGTT
AGTTGGTGGGGTAAAGGCTTACCAAGACGATGATGCATAGCCGAGTTGAGAGACTGATCGGCCACATTGGGAC
TGAGACACGGCCCAAACTCCTACGGGAGGCTGCAGTAGGGAATCTTCCACAATGGGCGCAAGCCTGATGGAGC
AACGCCGCGTGTGTGATGAAGGCTTTCGGGTCGTAAAGCACTGTTGTATGGGAAGAAATGCTAAAATAGGGAA
TGATTTTAGTTTGACGGTACCATACCAGAAAGGGACGGCTAAATACGTGCCAGCAGCCGCGGTAATACGTATG
TCCCGAGCGTTATCCGGATTTATTGGGCGTAAAGCGAGCGCAGACGGTTGATTAAGTCTGATGTGAAAGCCCG

GAGCTCAACTCCGGAATGGCATTGGAAACTGGTTAACTTGAGTGTTGTAGAGGTAAGTGGAACTCCATGTGTA

GCGGTGGAATGCGTAGATATATGGAAGAACACCAGTGGCGAAGGCGGCTTACTGGACAACAACTGACGTTGAG

GCTCGAAAGTGTGGGTAGCAAACAGGATTAGATACCCTGGTAGTCCACACCGTAAACGATGAATACTAGGTGT

TTAGGAGGTTTCCGCCTCTTAGTGCCGAAGCTAACGCATTAAGTATTCCGCCTGGGGGAGTACGACCGGCAAG

GTTGAAACTCAAAGGAATTGACGGGGGACCCGCACAAGCGGTGGAAGCATGTGGTTTTAATTCGAAGCAACGC

GAAGAACCTTACCAGGTCTTGACATCCTTTGAAGCTTTTAGAAGATAGAAGTGTTCTCTTCGGAGACAAAGTGA

CAGGTGGGTGCATGGGTCGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCT

TATTGTTAGTTGCCAGCATTCAGTTGGGCACTCTAGCGAGACTGCCGGTGACAAACCGGAGGAAGGCGGGGAC

GACGTCAGATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGCGTATACAACGAGTTGCCAA

CCTGCGAAGGTGAGCTAATCTCTTAAAGTACGTCTCAGTTCGGACTGCAGTCTGCAACTCGACTGCACGAAGT

CGGAATCGCTAGTAATCGCGGATCAGCACGCCGCGGTGAATACGTTCCCGGGTCTTGTACACACCGCCCGTCA

CACCATGGGAGTTTGTAATGCCCAAAGCCGGTGGC

12) Strain FM-S16 (1,470 bp)

TCGGATGAACGCGGCGGCGTGCCTAATACATGCAAGTCGAACGCACAGCGAAAGGTGCTTGCACCTTTCAAGT

GAGTGGCGAACGGGTGAGTAACACGTGGACAACCTGCCTCAAGGCTGGGGATAACATTTGGAAACAGATGCTA

ATACCGAATGAAACTTAGTGTCGCATGATACGAAGTTAAAAGGCGCTTTGGCGTCACCTAGAGATGGATCCGC

GGTGCATTAGTTAGTTGGTGGGGTAAAGGCCTACCAAGACAATGATGCATAGCCGAGTTGAGAGACTGATCGG

CCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCTGCAGTAGGGAATCTTCCACAATGGGCGAAA

GCCTGATGGAGCAACGCCGCGTGTGTGATGAAGGCTTTCGGGTCGTAAAGCACTGTTGTATGGGAAGAACAGC

TAGAATAGGGAATGATTTTAGTTTGACGGTACCATACCAGAAAGGGACGGCTAAATACGTGCCAGCAGCCGCG

GTAATACGTATGTCCCGAGCGTTATCCGGATTTATTGGGCGTAAAGCGAGCGCAGACGGTTGATTAAGTCTGA

TGTGAAAGCCCGGAGCTCAACTCCGGAATGGCATTGGAAACTGGTTAACTTGAGTGCAGTAGAGGTAAGTGGA

ACTCCATGTGTAGCGGTGGAATGCGTAGATATATGGAAGAACACCAGTGGCGAAGGCGGCTTACTGGACTGTA

ACTGACGTTGAGGCTCGAAAGTGTGGGTAGCAAACAGGATTAGATACCCTGGTAGTCCACACCGTAAACGATG

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AACACTAGGTGTTAGGAGGTTTCCGCCTCTTAGTGCCGAAGCTAACGCATTAAGTGTTCCGCCTGGGGAGTAC

GACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAG

CAACGCGAAGAACCTTACCAGGTCTTGACATCCTTTGAAGCTTTTAGAGATAGAAGTGTTCTCTTCGGAGACA

AAGTGACAGGTGGTGCATGGTCGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAA

CCCTTATTGTTAGTTGCCAGCATTCAGATGGGCACTCTAGCGAGACTGCCGGTGACAAACCGGAGGAAGGCGG

GGACGACGTCAGATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGCGTATACAACGAGTTG

CCAACCCGCGAGGGTGAGCTAATCTCTTAAAGTACGTCTCAGTTCGGATTGTAGTCTGCAACTCGACTACATG

AAGTCGGAATCGCTAGTAATCGCGGATCAGCACGCCGCGGTGAATACGTTCCCGGGTCTTGTACACACCGCCC

GTCACACCATGGGAGTTTGTAATGCCCAAAGCCGGTGGCCTAACCTTTTAGGAAGGAGCCGTCTAAGGCAGGA

CAGATGACTG

13) Strain BGM-S9 (1,382 bp)

GATCTGACGAGCTTGCTCTGATTTGATTTTATCTGACAAAGAGTGGCGAACGGGTGAGTAACACGTGGGTAAC

CTACCTCTTAGCAGGGGATAACATTTGGAAACAAGTGCTAATACCGTATAATACCAACAACCGCATGGTTGTT

GGTTGAAAGATGGTTCTGCTATCACTAAGAGATGGACCCGCGGTGCATTAGCTAGTTGGTAAGGTAACGGCTT

ACCAAGGCAATGATGCATAGCCAAGTTGAGAGACTGATCGGCCACAATGGGACTGAGACACGGCCCATACTCC

TACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGGCGCAAGCCTGATGGAGCAACGCCGCGTGTGTGATGA

AGGGTTTCGGCTCGTAAAACACTGTTATAAGAGAAGAACGGCACTGAGAGTAACTGTTCAGTGTGTGACGGTA

TCTTACCAGAAAGGAACGGCTAAATACGTGCCAGCAGCCGCGGTAATACGTATGTTCCAAGCGTTATCCGGAT

TTATTGGGCGTAAAGCGAGCGCAGACGGTTATTTAAGTCTGAAGTGAAAGCCCTCAGCTCAACTGAGGAATGG

CTTTGGAAACTGGATGACTTGAGTGCAGTAGAGGAAAGTGGAACTCCATGTGTAGCGGTGAAATGCGTAGATA

TATGGAAGAACACCAGTGGCGAAGGCGGCTTTCTGGACTGTAACTGACGTTGAGGCTCGAAAGTGTGGGTAGC

AAACAGGATTAGATACCCTGGTAGTCCACACCGTAAACGATGAGTGCTAGATGTTCGAGGGTTTCCGCCCTTG

AGTGTCGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGAC

GGGGACCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACAT

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CCCTTGCTAATCCTAGAAAAGGACGTTCCCTTCGGGACAAGGTGACAGTGGTGCATGGTTGTCGTCAGCTCGT

GTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATTATTAGTTGCCAGCATTCAGTTGGGCAC

TCTAGTGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTG

GGCTACACACGTGCTACAATGGCATATACAACGAGTCGCCAACCCGCGAGGGTGCGCTAATCTCTTAAAGTAT

GTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGTCGGAATCGCTAGTAATCGCGGATCAGCACGC

CGCGGTGAATACGTTCCCGGGTCTTGTACACACCGCCCGTCACACCATGAGAGTTTGTAACACCCAAAG

14) Strain FM-B16 (1,370 bp)

TTGAAGAGCTTGCTCAGATATGACGATGGACATTGCAAAGAGTGGCGAACGGGTGAGTAACACGTGGGAAACC

TACCTCTTAGCAGGGGATAACATTTGGAAACAGATGCTAATACCGTATAACAATAGCAACCGCATGGTTGCTA

CTTAAAAGATGGTTCTGCTATCACTAAGAGATGGTCCCGCGGTGCATTAGTTAGTTGGTGAGGTAATGGCTCA

CCAAGACGATGATGCATAGCCGAGTTGAGAGACTGATCGGCCACAATGGGACTGAGACACGGCCCATACTCCT

ACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGGCGAAAGCCTGATGGAGCAACGCCGCGTGTGTGAGAAG

GGTTTCGGCTCGTAAAACCTGTTGTAAGAGAAGAATGACATTGAGAGTAACTGTTCAATGTGTGACGGTATCT

TACCAGAAAGGAACGGCTAAATACGTGCCAGCAGCCGCGGTAATACGTATGTTCCAAGCGTTATCCGGATTTA

TTGGGCGTAAAGCGAGCGCAGACGGTTATTTAAGTCTGAAGTGAAAGCCCTCAGCTCAACTGAGGAATTGCTT

TGGAAACTGGATGACTTGAGTGCAGTAGAGGAAAGTGGAACTCCATGTGTAGCGGTGAAATGCGTAGATATAT

GGAAGAACACCAGTGGCGAAGGCGGCTTTCTGGACTGTAACTGACGTTGAGGCTCGAAAGTGTGGGTAGCAAA

CAGGATTAGATACCCTGGTAGTCCACACCGTAAACGATGAGTGCTAGGTGTTTGAGGGTTTCCGCCCTTAAGT

GCCGCAGCTAACGCATTAAGCACTCCGCCGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGG

GACCGCACAAGCGGTGGACAGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCCTTG

ACACTCCGAATGGAGCGTTCCCTTCGGGGACAAGGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTG

AGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATTACTAGTTGCCAGCATTTAGTTGGGCACTCTAGT

GAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTAC

ACACGTGCTACAATGGCGTATACAACGAGTTGCCAACCCGCGAGGGTGAGCTAATCTCTTAAAGTACGTCTCA

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GTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGTCGGAATCGCTAGTAATCGCGGATCAGCACGCCGCGGT

GAATACGTTCCCGGGTCTTGTACACACCGCCCGTCACACCTTGAGAGTTTGTAACACC

15) Strain P4-3 (1,380 bp)

ACATTGCAAAGAGTGGCGAACGGGTGAGTAACACGTGGGAAACCTACCTCTTAGCAGGGGATAACATTTGGAA

ACAGATGCTAATACCGTATAACAATGACAACCGCATGGTTGTTATTTAAAAGATGGTTCTGCTATCACTAAGAG

ATGGTCCCGCGGTGCATTAGCTAGTTGGTAAGGTAATGGCTTACCAAGGCGATGATGCATAGCCGAGTTGAGA

GACTGATCGGCCACAATGGGACTGAGACACGGCCCATACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACA

ATGGGCGAAAGCCTGATGGAGCAACGCCGCGTGTGTGATGAAGGGTTTCGGCTCGTAAAACACTGTTGTAAGA

GAAGAATGACCATTGAGGAGTAAACTGTTTCAAATGTTGTGACCGGTATTCTTACCCAGAAAAGGAAACGGGC

TAAATAACGTGCCCAGCAGCCCGCGGTAATTACGTATGTTTCCAAGCGTTATCCCGGATTTTATTGGGGCGTA

AAGCGAGCGCAGACGGTTATTTAAGTCTGAAGTGAAAGCCCTCAGCTCAACTGAGGAATTGCTTTGGAAACTG

GATGACTTGAGTGCAGTAGAGGAAAGTGGAACTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAAGAACA

CCAGTGGCGAAGGCGGCTTTCTGGACTGTAACTGACGTTGAGGCTCGAAAGTGTGGGTAGCAAACAGGATTAG

ATACCCTGGTAGTCCACACCGTAAACGATGAGTGCTAGGTGTTTGAGGGTTTCCGCCCTTAAGTGCCGCAGCT

AACGCATTAAGCACTCCGCCTGGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGACCCGCA

CAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCCTTGACAAC

TCCAGAAGATGGAGCGTTCCCTTCGGGGACAAGGTGACAGGTGGGTGCATGGGTTGTCGTCCACCTCCTGTCG

TGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATTACTAGTTGCCAGCATTCAGTTGGGCACTCTA

GTGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCT

ACACACGTGCTACAATGGCGTATACAACGAGTTGCCAACCCGCGAGGGTGAGCTAATCTCTTAAAGTACGTCT

CAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGTCGGAATCGCTAGTAATCGCGGATCAGCACGCCGCG

GTGAATACGTTCCCGGGTCTTGTACACACCGCCCGTCACACCATGAGAGTTTGTAACACCCAAAG

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16) Strain Ru20-1

ACGAACGTTGGCGGCGTGCCTAATACATGCAAGTCGAACGCGGTCTCCTAATTGACAAACCGTGCTTGCACGG

GTTGGATTTTAGATCGGACCGAGTGGCGAACTGGTGAGTAACACGTGGGTAACCTGCCCAGAAGTAGGGGATA

ACACCTGGAAACAGATGCTAATACCGTATAACAACTAAAACCACATGGTTTTAGTTTGAAAGCTGGCCTTGGTG

CTAGTGCTTTTGGATGGACCCGCGGCGTATTAGCTAGTTGGTGAGATAATAGCTCACCAAGGCGATGATACGT

AGCAGACCTGAGAGGGTAATCTGCCACAATGGGACTGAGACACGGCCCATACTCCTACGGGAGGCAGCAGTA

GGGAATCTTCCACAATGGACGCAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGGTTTCGGCTCGTAAA

ACTCTGTTGTTAGAGAAGAACGACTGTGAGAGCAACTGCTCACGGCGTGACGGTATCTAACCAGAAAGTCACG

GCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAACGTTGTCCGGATTTATTGGGCGTAAAGCGA

GCGCAGGCGGTTTTTTAAGTCTGATGTGAAAGCCTTCGGCTTAACCGAAGAAGTGCATCGGAAACTGGGAAAC

TTGAGTGCAGAAGAGGACAGTGGAACTTCATGTGTAGCGGTGAAATGCGTAGATATATGAAGGAACACCAGTG

GCGAAGGCGGCTGTCTAGTCTGCATCTGACGCTGAGGCTCGAAAGCATGGGTAGCAAACAGGATTAGATACCC

TGGTAGTCCATGCCGTAAACGATGAATGCTAGGTGTTGGGAGGTTTCCGCCTCTCAGTGCCGGAGCTAACGCA

TTAAGCATTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGACCCGCACAAGCGG

TGGAGCATGTGGTTTAATTCGATGCTACGCGAAGAACCTTACCAGGTCTTGACATCTTCTGTTAGCCTAAGAG

ATTAGGTGTCCCCTTCGGGGGCAGAATGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGG

GTTAAGTCCCGCAACGAGCGCAACCCTTGTCTTTAGTTGCCAGCATTCAGTTGGGCACTCTAGAGAGACTGCC

GGTGATAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCT

ACAATGGACGGTACAACGAGTTGCGAAACCGCGAGGTCAAGCTAATCTCTTAAAGCCGTTCTCAGTTCGGATT

GCAGGCTGCAACTCGCCTGCATGAAGTTGGAATCGCTAGTAATCGTGGATCAGCATGCCACGGTGAATACGTT

CCCGGGTCTTGTACACACCGCCCGTCACACCATGAGAGTTTGTAACACCCAAAGTCGGTTGGATAACCGTTAG

GAGTCCGCCGCCTAA

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3) *PheS* gene sequences of a novel species

ATGCAGGACACGTTCTACATCACGAAGGAAATTTTAATGCGGACGCATACTTCGCCCATGCAAGCTCGGGCGT

TAGAAACCCACGATTTTTCTACGGGACCTTTGAAAATGATTTCTCCAGGAGTGGTTTTTCGGCGGGATACTGAT

GATCCGACTCATTCGCACCAGTTCCATCAAGTTGAAGGAATTGTCATTGACAAACACATTACGATGGCCGATCT

CAAGGGGACCTTGGCAGCCATGACGCACGCGTTATTTGGATCCAAGTTTGCGGTCCGGCTTCGCCCGAGTTAT

TTCCCATTTACAGAACCATCGGTCGAAGCCGACATTACCTGCATGAATTGTGGTGGCAAGGGCTGTTCAGTTT

GTAAGGGTTCCGGCTGGATCGAAGTCTTAGGAGCCGGCTGGGTCCACCCA

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PUBLICATIONS

1. Techo, S., Visessanguan, W., Vilaichone, R., Tanasupawat, S. 2018. Characterization and antibacterial activity against Helicobacter pylori of lactic acid bacteria isolated from Thai fermented rice noodle. Probiotics and Antimicrobial Proteins. https://doi.org/10.1007/s12602-018-9385-z

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2. Techo, S., Visessanguan, W. and Tanasupawat, S. 2017. Probiotic property and anti-Helicobacter pylori activity of plant-derived lactic acid bacteria. RRi Congress III, July 24, Queen Sirikit National Convention Center, Bangkok, Thailand