Screening of Probiotic Lactic Acid Bacteria Isolated from Fermented Pak-Sian and Its Application as a Starter Culture

Supaporn Pumriw

A Thesis Submitted in Partial Fulfillment of Requirements for
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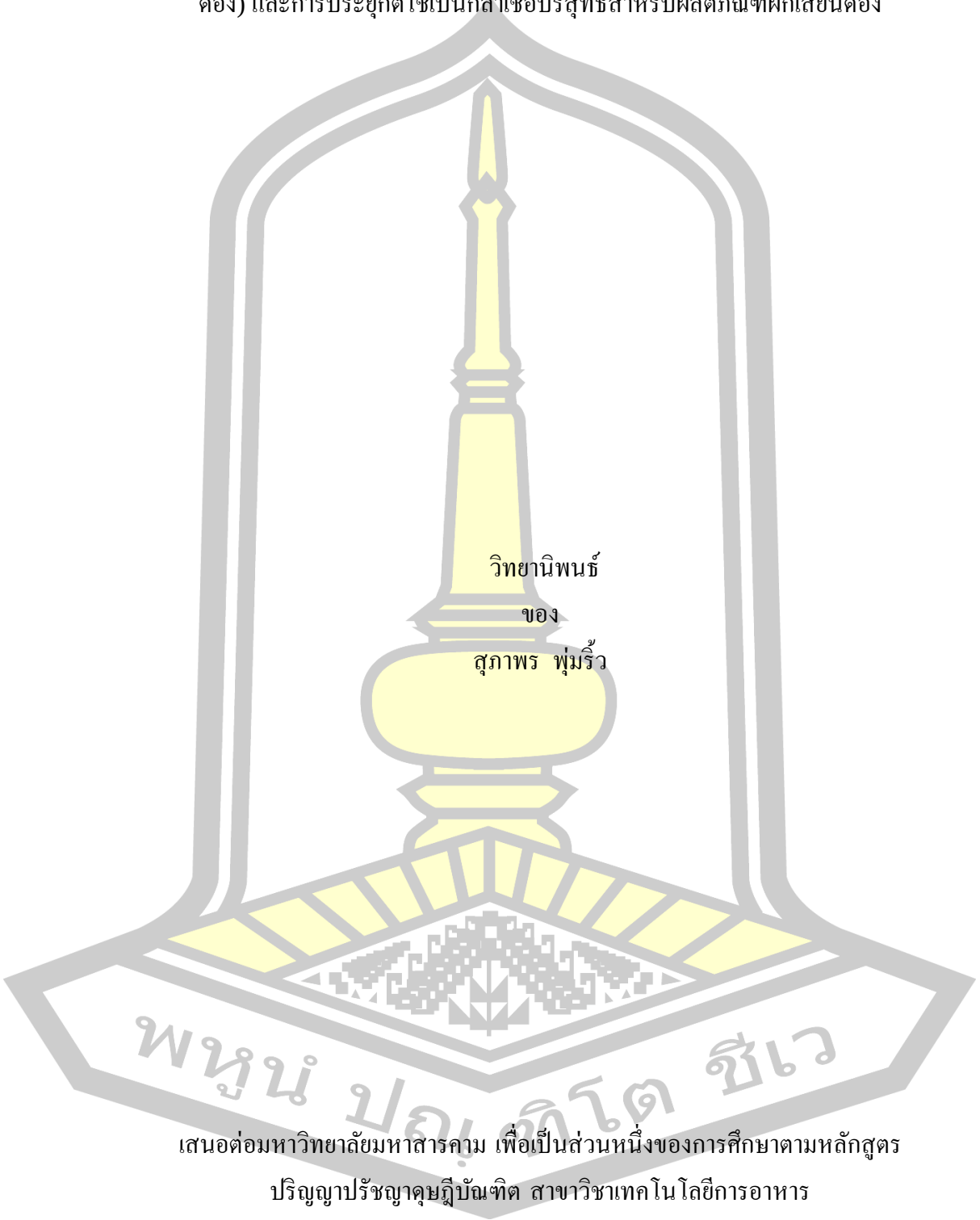
การคัดแยกและคัดเลือกโปรไบโอติกแบคทีเรียแลคติกจากอาหารหมักพื้นบ้านของไทย (ผักเสี้ยนดอง) และการประยุกต์ใช้เป็นกล้าเชื้อบริสุทธิ์สำหรับผลิตภัณฑ์ผักเสี้ยนดอง



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ABSTRACT

Fermented Pak-Sian is commonly consumed in the northeastern parts of Thailand and is rich in lactic acid bacteria (LAB). However, each region has a different fermentation recipe to ferment Pak-Sian which may affect the diversity of LAB. This research aimed to isolate and identify LAB from fermented Pak-Sian samples, to determine their probiotic properties and to use probiotic starter culture for fermentation of Pak-Sian. The samples were collected from local markets in Kalasin, Sakon Nakhon, Khon Kaen and Maha Sarakham Provinces. LAB were isolated on MRS agar supplemented with bromocresol purple (0.05%) as a pH indicator. The putative LAB were selected based on the yellow zone surrounding the colony, catalase-negative and gram-positive characters. A total of 234 presumptive LAB were selected. These presumptive LAB were grouped using whole-cell protein patterns by SDS-PAGE. The results presented 61 presumptive LAB from 8 local markets in this study. These LAB were confirm and identified by 16S rDNA analysis. LAB identified 17 isolates as follows *Pediococcus pentosaceus* (KS12, KS218, KS230, SK337, MK74), *Pediococcus* sp. KS215 *Lactobacillus plantarum* (SK321, KK53, KK518, MK711, MK724), *Lactobacillus brevis* (SK335), *Lactobacillus fermentum* (SK324, SK48, SK434), *Weissella cibraria* (SK415, SK432). The selected strains were investigated for probiotic properties namely bile salt tolerance (0.3% bile salt), pH tolerance (pH 2.5), survival in simulated gastric and intestinal tract, antimicrobial activity (*Bacillus cereus*, *Staphylococcus aureus*, *Samonella typhymurium* and *Escherichia coli*) antibiotic susceptibility (streptomycin, rifampicin, vancomycin, ampicillin, azithromycin and chloramphenicol), biogenic amine production and haemolytic activity. The results found that 14 strains showed resistance to the pH 2.5 and all LAB isolates showed resistance to bile salt. High survival rates of LAB in simulated gastric and intestinal tract indicated resistance to simulated gastric and intestinal tract. LAB demonstrated antimicrobial activity by inhibiting the pathogenic bacteria indicator. Moreover, most LAB strains were resistant to all antibiotics tested and some *Lactobacillus* strains were moderately susceptible to cholamphenical, rifampicin and ampicillin. All 14 strains did not produce biogenic amine., Only 8 out of the selected 17 strains showed no haemolysis activity (g-haemolysis) and therefore were selected for mucin adhesion capacity

determination. Three strains (*P. pentosaceus*, *Lb. fermentum* and *Lb. brevis*) showed 0.03-2.39% adhesion. *Lb. fermentum* (SK324) and *Lb. brevis* (SK335) showed high percentage of adhesion capacity (2.39% and 2.34%, respectively). Therefore, it was chosen to be used as a starter culture for fermentation of fermented Pak-Sian. Fermented Pak-Sian was subjected to 4 treatments: *Lb. fermentum* (SK324), *Lb. brevis* (SK 335), mixed culture *(Lb. fermentum* (SK324); *Lb. brevis* (SK 335); 1:1) and compared with control (no add starter culture). In this study, 10^6 CFU/ml of initial starter culture was used and fermented for 3 days. The results showed that pH value of starter culture was lower than the control treatment The lactic acid bacteria (LAB) count and total plate count (TPC) showed an increase of up to 10^6 CFU/g during the fermentation of Pak-Sian of up to which is an added advantage to consumer health. The presence of short chain fatty acid indicated that all the treatments produced acetic acid while propionic acid and butyric acid were not found after fermentation. The sensory evaluation acceptance scores obtained during the utilization of starter culture and control treatment were not significant (P>0.05) in terms of colour, smell, taste, texture and overall acceptance. Therefore, these strains may be used as an alternative starter culture to produce fermented Pak-Sian thereby leading to an improvement in product development and overall increase in potential health benefits.

Keyword : Lactic acid bacteria, SDS-PAGE, Whole-cell protein, Fermented Pak-Sian, Probiotic

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CHAPTER I
INTRODUCTION

1.1 Background

Nowadays, consumers are becoming more and more interested in health care. The functional foods receive more attention and there are increasing number of studies about the healthy foods which supposedly support consumers health. Probiotics are living microorganisms in food which confers advantages to the health of consumers by keeping or improving their gut microbial balance upon consuming in appropriate doses (Butel, 2014; W. H. Holzapfel, Haberer, Snel, Schillinger, & in't Veld, 1998; Jakubczak, Stachelska, Świsłocka, & Lewandowski, 2012a; Kos *et al*., 2008). It can be seen that the consumption of the probiotic product is increasing and continues to grow (Kumar, Vijayendra, & Reddy, 2015; Šušković *et al*., 2010). The advantages of probiotic products are as follows: prevention of diarrhea, management of the stomach and gastrointestinal infections, management of chronic inflammation, and decreasing level of cholesterol (Ewaschuk & Dieleman, 2006; Jankovic, Sybesma, Phothirath, Ananta, & Mercenier, 2010; Ooi & Liong, 2010; Wang *et al*., 2014). Usually, probiotics have been supplemented in yoghurt and fermented dairy products. However, probiotics are increasingly used in non-dairy products as well due to the increasing trend of veganism and increasing number of customers with lactose intolerance and cholesterol-controlled consumers (Martins *et al*., 2013). Thus, the development of non-dairy probiotic products mainly made from plants is an alternative for vegetarian consumers who have the restriction in consuming dairy products (Martins *et al*., 2013; Peres, Peres, Hernández-Mendoza, & Malcata, 2012; Rivera-Espinoza & Gallardo-Navarro, 2010). Moreover, the plants have higher dietary fibers, vitamins, minerals and antioxidants than dairy sources (H. Lee *et al*., 2011; Yoon, Woodams, & Hang, 2004). There are several research studies on the probiotics from fermented plant products (S.-M. Chang, Tsai, Wee, & Yan, 2013; Karasu, Şimşek, & Çon, 2010; K. W. Lee *et al*., 2016). It can be seen that the fermented products from plants provide a source of lactic acid bacteria (LAB) with probiotic property. Therefore, the development of probiotic products from vegetables is an alternative for consumers with a restricted diet preference and allergenic food.

The advantage of consuming the fermented vegetable product is that it lacks heat processing of food, and thus a greater amount of live lactic acid bacteria can survive and pass into the human gut.

Pak-Sian is a local vegetable of Thailand. Pak-Sian is a highly nutritious vegetable containing vitamin A (6.7-18.9 mg), vitamin C (127-484 mg), mineral (calcium 213-434 mg and potassium 410 mg) phenolic compounds (520-910 mg) found in the leaves, which contributes to an acerbity taste of the vegetable (S. Mishra, Moharana, & Dash, 2011). Pak-Sian is commonly consumed in the fermented form as fermented Pak-Sian rather than the fresh vegetable because the fresh vegetable has unpleasant odor and contains hydro cyanide which has a toxic effect on the central neuron system (Pillai & Nair, 2013). Fermented Pak-Sian is commonly consumed in the North Eastern region of Thailand. It is a very common pickled leafy vegetable and the preparation as side-dish is simply made with low cost. The fermentation of Pak-Sian in each region of in the North Earthern region of Thailand has a different fermentation recipes such as different salt concentration, fermented rice water and using varieties of Pak-sian such as *Cleome gynandra* L. (Pak-Sian-Baan), *Cleome chelidonii* L. (Pak-Sian-Bah), *Cleome viscosa* L. (Pak-Sian-Phee) and *Cleome rutidosperma* DC. (Pak-Sian-Muang) from different origins of Pak-Sian which may affect the diversity of LAB in fermented Pak-Sian. Thus, fermented Pak-Sian was collected for the study from different regions in the North Earthern region of Thailand to increase the possibility of finding new LAB species with probiotic properties. The isolation and identification of LAB from fermented Pak-Sian was carried out as per previous studies that discovered LAB such as *Lactobacillus brevis*, *Pediococcus cerevisiae*, *Lactobacillus plantarum* (Pillai & Nair, 2013; S Tanasupawat & Komagata, 1995), *Lactobacillus pentosus* (Somboon Tanasupawat, 2009; Somboon Tanasupawat *et al*., 1992) and *Pediococcus pentasaceus* (Somboon Tanasupawat & Daengsubha, 1983) which were identified during fermentation of fermented Pak-Sian. To date, probiotic properties of LAB from fermented Pak-Sian is yet to be reported. Hence, our aim is to isolating and identifying probiotic LAB from fermented Pak-Sian of Thailand origin and use the best LAB with most fulfilled probiotic properties as a starter culture to make the better health-promoting fermented Pak-Sian product for Thai consumers

with consistent quality due to controlled fermentation, better desirable sensory attributes and abundant with probiotic bacteria that are beneficial to human health. Therefore, the objectives of this study are as follows; (i) To isolate and screen for LAB from fermented Pak-Sian and (ii) To study probiotic properties of isolated LAB bacteria and (iii) To evaluate sensory evaluaton, chemical and microbiological properties study of fermented Pak-Sian products fermented by isolated bacteria as probiotic starter culture in single culture and binary culture.

1.2 The objectives of the research

1.2.1 To isolate and screen for LAB from fermented Pak-Sian

1.2.2 To determine the probiotic properties of isolated LAB bacteria

1.2.3 To apply probiotic obtained from this study to prepare fermented Pak-Sian and to evaluate sensory evaluation, chemical and microbiological quality of the products

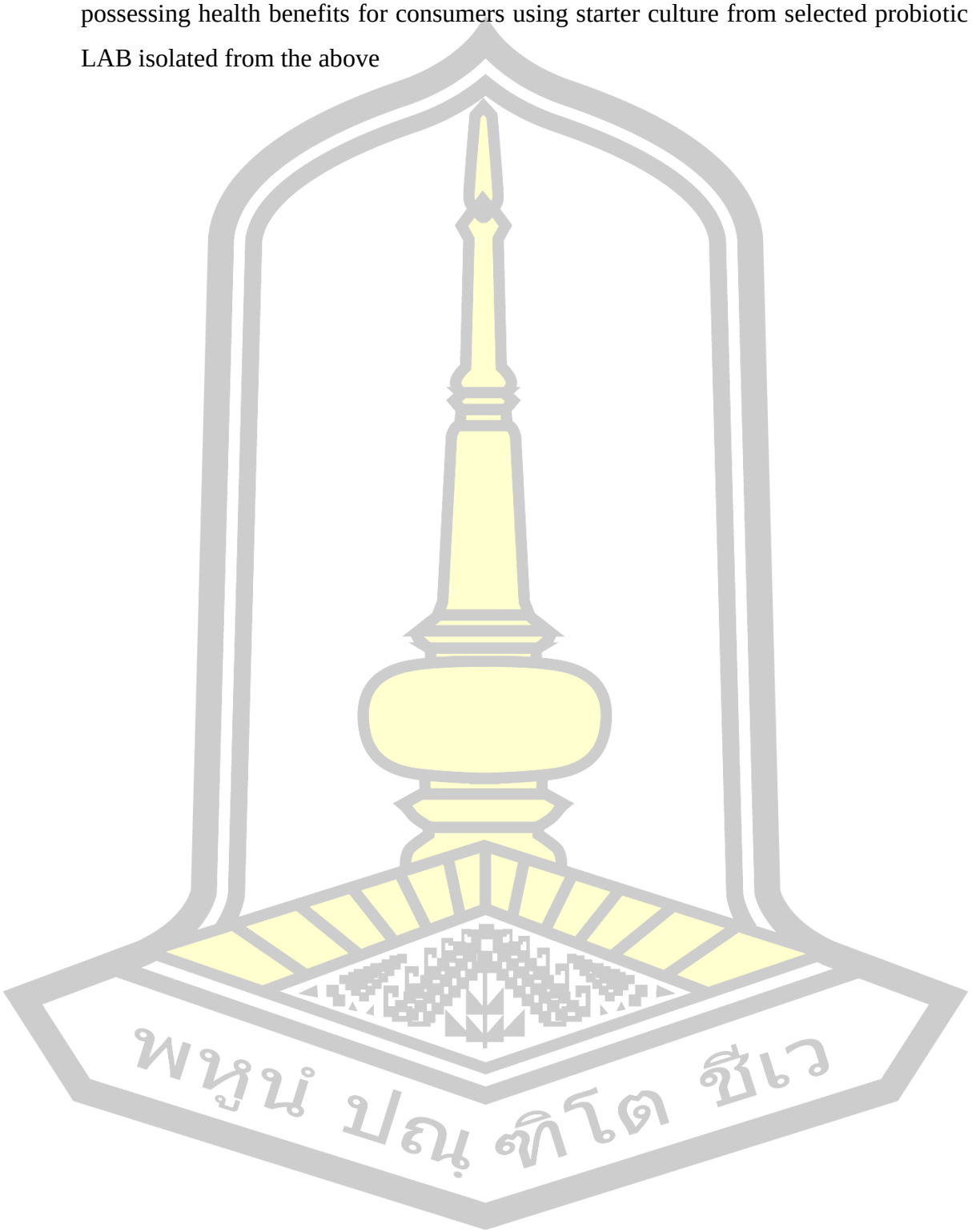
1.3 The scope of the research

The fermented Pak-Sian in this study was purchased and sampled from 8 local markets in Kalasin, Sakon Nakhon, Maha Sarakham and Khon Kaen province. The isolation of LAB and screening for LAB was performed based on bacterial characteristics of LAB such as colony appearance, gram-positive bacteria, bacillus shaped and negative for catalase enzyme test. The isolated LAB was identified using 16S rDNA gene. Isolated LAB was studied for probiotic properties including pH tolerance, bile salt tolerance, survival in simulated gastrointestinal tract condition, adhesion capacity, biogenic amine production, antibiotic susceptibility, haemolytic activity, antimicrobial activities and the short chain fatty acid. The best LAB with most fulfilled probiotic properties was used as probiotic starter culture in single culture and binary culture in Pak-Sian fermentation to quality control of product compare to the control fermentation.

1.4 The expected outcomes

1.4.1 To obtain the LAB species with probiotic properties

1.4.2 To obtain fermented Pak-Sian product with consistent quality, safe and possessing health benefits for consumers using starter culture from selected probiotic LAB isolated from the above



CHAPTER II
LITERATURE REVIEW

2.1 Pak-Sian (*Cleome gynandra*)

The scientific name of Pak-Sian is *Cleome gynandra*. Pak-Sian is the common and local name in Thailand (Chweya & Mnzava, 1997; Neamsuvan & Bunmee, 2016). Pak-Sian present in Thailand belongs to 4 types including the *Cleome gynandra* L. (PakSian-Baan), *Cleome chelidonii* L. (Pak-Sian-Bah), *Cleome viscosa* L. (Pak-Sian-Phee) and *Cleome rutidosperma* DC. (Pak-Sian-Muang). Pak-Sian is the common and the local name in Thailand (Chweya & Mnzava, 1997; Neamsuvan & Bunmee, 2016). Nutritional composition of the leaves of *Cleome gynandra L*. is shown in Table 1. The nutrition value may vary with soil fertility, plant type, environment, plant age. The *C. gynandra* is a source of high nutrient vegetable, especially vitamins (A and C) and minerals (calcium and iron). The leaves contain phenolic compounds, which give the vegetable an astringent taste (Chweya & Mnzava, 1997).

The leaves of Pak-Sian present phytochemical compounds such as *C. gynanda* L. were found to be carotenoids, cardiac glycosides, cyanogenetic glycoside, flavonoids phenol, saponins, sugars, tannins, triterpenes and alkaloids and antroguinone (Anbazhagi, Kadavul, Suguna, & Petrus, 2009). Similarly, the leaves of *C. viscosa* L. were found terpeniod, phenolic, morphine alkaloids, proteins, starch and triterpenes (Jane & Patil, 2012; Pillai & Nair, 2013) and *C. chelidonii* L. were found terpenoids, saponins, tannins, sugar, phenolics, flavonoids, cardiac glycosides, anthroquinone, reducing sugar, starch, emodol, steroid, vitamin C, proteins, free amino acids, carbohydrates, glycosides and alkaloids (Sumitha & Gurulakshmi, 2015). The antioxidant activity of *C. gynanda* L. leaf was found to be 523.67±4.16 mg vitamin C equivalents/100g, polyphenol 321±2.65 mg gallic acid equivalents/100 g, flavonoids 207±4.58 mg quercetin equivalents/100 g powdered leaf material (Anbazhagi *et al*., 2009) and Sumitha and Gurulakshmi (2015) found the leaf extract of *C. chelidonii L.* revealed strong free radical scavenging and antioxidant activities.

The antioxidant properties, especially the polyphenolic have significant effects on human carcinogenesis and cardiovascular diseases (Arts & Hollman, 2005). Furthermore, the leaves can inhibited microorganisms, according to Williams, Vasques, Reid, Porter, and Kraus (2003) studied on biological activities of leaf extract from *C. viscosa* L., the results indicated the leaf extract contain a 14-member ring, cembranoid diterpene, which effect on *Bacillus subtilis* (gram positive) and *Pseudomonas flurescens* (gram negative) and did not inhibit the growth of the fungus *Cladosporium cucumerrium*. The antimicrobial activity of *C. viscosa* L. can inhibited *K. pneumonia*, *S. aureus*, *P. aeruginosa*, *S. pneumoniae* and *E. coli* (Jane & Patil, 2012). Samy, Ignacimuthu, and Raja (1999) demonstrated that the extract of *C. viscosa* L. and *C. gynandra* L. (concentration 30 and 40 mg/ml) inhibited *Aeromonas hydrophila* and *Bacillus cereus*. The flavone glycoside from flower of *C. viscosa* L. was Quercetin 3-0-2($2^{//}$acetyl)-glucoside which inhibited *Staphylococcus aureus* (gram positive), *Escherichia coli* (gram negative) and revealed anti-inflammatory activity of rat paw edema (Senthamilselvi, Kesavan, & Sulochana, 2012).

Table **1** Nutritional and chemical composition of *Cleome gynandra* leaves (% or mg/100g edible parts)

Nutrient	Range of value
Moisture content (%)	81.8-89.6
pH	5.8
Crude protein (%)	3.1-7.7
Crude fiber (%)	1.3-1.4
Carbohydrates (%)	4.4-6.4
Ether extract (%)	0.4-0.9
Total ash (%)	2.1-3.0
Potassium (mg)	410
Calcium (mg)	213-434
Magnesium (mg)	86
Sodium (mg)	33.6
Phosphorus (mg)	12
Iron (mg)	1-11
Zinc (mg)	0.76
Copper (mg)	0.46
β-carotene (mg)	6.7-18.9
Ascorbic acid (mg)	127-484
Oxalate (mg)	8.8
Total phenolics (mg)	520-910

Source: Chweya and Mnzava (1997)

2.2 Lactic acid bacteria

Lactic acid bacteria (LAB) are defined by the bacteria organism that could produce lactic acid as a sole or main end-product of carbohydrate metabolism. LAB are gram-positive bacteria, non-sporing, non-motile, cocci or rods shape, catalase negative, anaerobic, micro-aerophilic or aero-tolerant, acid tolerant and ability to ferment carbohydrates to produce the major lactic acid (Wood & Holzapfel, 1992). LAB are chemotropic, they find the energy required for their entire metabolism from

the oxidation of chemical compounds. LAB has limited biosynthetic capability and can be grown in nutritionally rich environments which are high in carbon and nitrogen source. For the identification of LAB, the DNA-based methods targeting genes such as 16S rRNA, are utilized to classify taxonomic of LAB. The polymerase chain reaction (PCR) based method is important for characterization of LAB strains. LAB includes the genera *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Steptococcus*, *Tetragenococcus*, *Vagococcus*, *Aerococcus* and *Weissella* (Stiles & Holzapfel, 1997). LAB are commonly related with plant and animal raw materials, and are prevalently found in fermented food products such as dairy (yogurt, cheese), meat (sausages), fish, vegetable (sauerkraut, kimchi,) and cereal plant as shown in Table 2. Some species found within humans and animal cavities including the gastro intestinal tract; *Lactobacillus acidophilus*, *Lactobacillus gasseri*, *Lactobacillus johnsonii*, *Lactobacillus plantarum*, *Steptococcus agalactiae*, *Enterococcus faecalis*. The oral cavity; *Streptococcus mutants*, *Bifidobacterium longum*, and the vaginal cavity; *B. longum*, *S. agalactiae*, *Lactobacillus crispatus* (Khalid, 2011; Klaenhammer, Barrangou, Buck, Azcarate-Peril, & Altermann, 2005).

LAB possess important physiological properties such as substrate utilization, metabolic capabilities and probiotic properties. They are used as a food preservative, leading to their widespread human consumption and they are considered as Generally Recognized as Safe (GRAS) microorganism (Klaenhammer *et al*., 2005; Silva, Carvalho, Teixeira, & Gibbs, 2002).

Table 2 Examples of fermented foods

Product	Country	Major ingredients	Microorganism	Appearance/usage	References
Kimchi	Korea	Cabbage, radish, vegetable, salt	*Lb. mesenteroides*, *Lb. plantarum*, *Lb.* sakei	Salad, side dish	K. W. Lee *et al.* (2016); Khan and Kang (2016b); H. Lee *et al.* (2011)
Dhamuoi	Vietnam	Cabbage, vegetable	*Lb. plantarum*, *Lb. brevis*, *Lb. fermentum*, *Lb. casei*, *Lb. rhamnous*	Salad, side dish	M.-E. Lee *et al.* (2015)
Nham	Thailand	Pork, salt, cooked, rice	*Lb. plantarum*, *Lb. brevis*, Lb. *fermentum* , *Lb. pentosus*	Pork meat in banana leaves	Klayraung, Viernstein, Sirithunyalug, and Okonogi (2008); Somboon Tanasupawat *et al.* (1992)
Takju	Korea	Rice, wheat	*Lb. paracasei*, *Lb. arizonensis*, *Lb. plantarum*, *Lb. harbinensis*, *Lb. parabuchneri*, *Lb. brevis*, *Lb. hilardii*	Turbid liquid	Jin, Kim, Jin, Eom, and Han (2008)

*Table **2*** Examples of fermented foods (continued)

Product	Country	Major ingredients	Microorganism	Appearance/usage	References
Idli	India, Sri Lanka	Rice, Black gum	*Leoconostoc* spp., *Weissella* spp., *Pediococcus* spp., *Lactococcus* spp.	Steamed cake	Saravanan, Gopu, and Shetty (2015)
Nem-chua	Vietnam	Meat	*Lb. plantarum*	Sausage	Nguyen, Elegado, Librojo-Basilio, Mabesa, and Dizon (2010)
Khanomjeen	Thailand	Rice	*Lb. plantarum, Lb. fermentum, P. acidilactici*	Noodle	Oupathumpanont, Chantarapanont, Suwonsichon, Haruthaithanasan, and Chompreeda (2009); (S Tanasupawat & Komagata, 1995)
Pla-ra	Thailand	shrimp, rice, salt shrimp, salt	*P. halophilus, S. piscifermentans, E. faecalis*	Side dish	S Tanasupawat and Komagata (1995)
Dakguadong	Thailand	mustard leaf	*Lb. plantarum*	Salad, salt side dish	S Tanasupawat and Komagata (1995)

2.3 Methods for identifying LAB

The identification method of LAB is down to the genus or species level is generally phenotypical and genotypical. This method is considered for its taxonomic resolution, intensive assignment, speed and cost. In general, the phenotypical method is less expensive in contrast to genotypical technique. Sometime, phenotypical methods have limitation such as poor reproducibility, the uncertainty of some techniques (frequently affecting the culture is the complex growth condition requirement), the extensive logistics for large-scale examination, and low discriminatory power (Mohania *et al*., 2008; Temmerman, Huys, & Swings, 2004). These disadvantages have an influence on the dependability and consistency of phenotypic technique for LAB identification down to the genus or species level. However, the genotypic methods have limitations such as cost, tools and database. Therefore, the polyphasic approach is now preferred which is a combination of the two aforementioned methods for identification. Table 3 is the lists of the identification techniques for identifying LAB. Several research groups used the phenotypical and genotypical basis for the identification of LAB. For example, Lee *et al*. (2016) identificatified probiotic LAB isolated from Kimchi by using API 50CHL and 16S rRNA gene sequencing. *Lb. plantarum* C182, *Leu. mesenteroides* C10, *Leu. mesenteroides* F27 and *Leu. mesenteroides* C4 were identified. Similarly, Nguyen *et al*. (2010) isolated and characterized LAB from a traditional fermented meat (Vietnam) by using API 50 CHL and 16S rRNA gene sequencing. The isolates found were identified as *Lb. plantarum* WCFS1.

*Table **3*** List of the identification techniques for LAB

Technique	Principle	Workload	Discriminatory power	Reproducibility
Phenotypic methods Morphological analysis	Microscopic analysis	Low	Genus level or less	Moderate
Biochemical characterization	Assimilation and fermentation patterns (API, BIOLOG)	Low	Genus or species level	Moderate
Physiological analysis	Growth characteristics, simple tests	Moderate	Genus level or less	Low
Genotyping method Specific primers	PCR with group-specific primers	Low	Depending on primer	High
RFLP	Restriction Enzyme Analysis (REA) of DNA or PCR amplicons	Moderate	Species to strain level	High
AFLP	Combination of REA and PCR amplication	High	Species to strain level	High
RAPD-PCR	Randomly primered PCR	Low	Strain level	Low
Rep-PCR	PCR targeting repetitive interspersed sequences	Low	Strain level	High
PFGE	REA and pulsed-field gel electrophoresis	High	Strain level	High
Sequencing	Determination of gene sequences (16S rDNA)	High	Genus to species level	High

Source: Temmerman *et al*. (2004)

2.3.1 Phenotypical characterization

The basis of LAB classification is phenotypical properties, such as morphology, glucose fermentation, growth at various temperatures, lactic acid arrangement, the fermentation of different carbohydrate, the methyl esters of fatty acids, and the form of proteins in the cell wall. Occasionally, these methods have limitation including poor reproducibility and identification at the genus level. According to Corsetti *et al*. (2001), it was examined that 317 LAB isolates from sourdoughs by using morphological and physiological test by using the API 50CHL kits, it was found 38% of LAB isolated were identified until the species level. Also, Mahasneh, Hamdan, and Mahasneh (2015) identified of bacteria strains from the local traditional fermented product from the API50 CHL kits, found that the identification of the 17 *Lactobacillus* isolates, 8 out of 17 isolated belong to *Lactobacillus plantarum*, 3 isolated belong to *Lactobacillus pentosus*, 2 isolated belong to *Lactobacillus* and 4 isolated were not designated to species.

2.3.2 Genotypical characterization

The genotypic technique is a tool for DNA-based identification. The advantage of these techniques is that there is no effect of the variation in growth of microorganism on the identification. The genotypic techniques can be used to differentiate from species level to strains level. The Polymerase Chain Reaction (PCR) is based on genotypic method, which is a technique used for amplification of specific targeted DNA fragments by using the oligonucleotide primers under PCR condition. The regions of 16S or 23S rDNA can be used for identification of various LAB (Khan & Kang, 2016a; K. W. Lee *et al*., 2016; Schleifer *et al*., 1995; J. P. Tamang, Tamang, Schillinger, Guigas, & Holzapfel, 2009; J. Yang *et al*., 2014). These regions are constant and highly reserved function and not variation by environmental conditions. The 16S rDNA gene is the most common gene region in the bacterial diversity investigation. The unknown bacteria isolates were identified by 16S or 23S rDNA sequencing. These regions sequences were compared with a DNA sequence from an online database such as EMBL (http://www.ebi.ac.uk/) and Genbank (http://www.ncbi.nlm.nih.gov/) database. Afterwards, the DNA sequences

were determined by percent similarity between DNA from databases by using BLAST or FASTA.

*Table **4*** List of PCR primers designed for identification and detection of LAB

Primer designation	Target organism (Target molecule)
Lac1/2	*Lactobacillus*/*Leuconostoc*/*Pediococcus*/*Weisella*
lm-26f/lm-3r	*Bifidobacterium* (16S rDNA)
Brif164/Bif662	*Bifidobacterium* (16S rDNA)
lnfY-BV.L/R	*Bifidobacterium infantis* (16S-23S rDNA)
BreY- BV. R /L	*Bifidobacterium breve* (16S-23S rDNA)
BiADO-1/2	*Bifidobacterium adolescentis* (16S rDNA)
BiANG1/2	*Bifidobacterium angulatum* (16S rDNA)
BiBlF1/2	*Bifidobacterium bifidum* (16S rDNA)
BiCATg1/2	*Bifidobacterium catenulatum* (16S rDNA)
BiLONg1/2	*Bifidobacterium longum/infantis* (16S rDNA)
LactV5	*Lactcoccus lactis* (16S rDNA)
LeucV5	*Leuconostoc mesenteroides* (16S rDNA)
LbpV3	*Lactobacillus plantarum* (16S rDNA)
Case1	*Lactobacillus casei* (16S rDNA)
Ferm1	*Lactobacillus fermentum* (16S rDNA)
Para1	*Lactobacillus paracasei* (16S rDNA)
Reut1	*Lactobacillus reuteri* (16S rDNA)
Sal1	*Lactobacillus salivariusi* (16S rDNA)
Aci-1/2	*Lactobacillus acidophilus* (16S-23S rDNA)
SS1-DB1	*Lactobacillus delbrueckii* (16S rDNA)
SS2-HE1	*Lactobacillus helveticus* (16S rDNA)
Y2-rham	*Lactobacillus rhamnosus* (16S rDNA)
Thl/Thll	*Streptococcus thermophiles* (16S-23S rDNA)
16MAC	*Streptococcus macedonius* (16S rDNA)
ENT1-ENT2	*Enterococcus* (16S rDNA)
AVI	*Enterococcus avium* (16S rDNA)
ASI	*Enterococcus asini* (16S rDNA)
CEC	*Enterococcus cecorum* (16S rDNA)
COL	*Enterococcus columbae* (16S rDNA)

*Table **4*** List of PCR primers designed for identification and detection of LAB (continued)

Primer designation	Target organism (Target molecule)
CGF	*Enterococcus casseliflavus/gallinarum/flavescens*(16S rDNA)
DUR	*Enterococcus durans* (16S rDNA)
DIS	*Enterococcus dispar* (16S rDNA)
Efs130c	*Enterococcus faecalis* (16S rDNA)
FMDUR	*Enterococcus faecium/durans* (16S rDNA)
HIR	*Enterococcus hirae* (16S rDNA)
MAL	*Enterococcus malodoratus* (16S rDNA)
MUN	*Enterococcus mundtii* (16S rDNA)
PSE	*Enterococcus pseudoavium* (16S rDNA)
RAF	*Enterococcus raffinosus* (16S rDNA)
SAC	*Enterococcus sacharolyticus* (16S rDNA)
SOIL	*Enterococcus solitatius* (16S rDNA)

Source: Temmerman *et al*. (2004)

2.4 Metabolic activity of lactic acid bacteria

Lactic acid bacteria (LAB) are generally mesophillic, grow at temperature of 5 -45 °C, pH 4.0-4.5, while some strains are grow at pH 9.6 and pH 3.2. The stains are generally weak proteolytic and lipolytic and require amino acids, purine and pyrimidine bases and vitamins B for growth (Caplice & Fitzgerald, 1999).

LAB can produce lactic acid from hexoses and they obtain energy through substrate level phosphorylation. The type of lactic acid produced during fermentation may be L(+) lactic acid, D(-) lactic acid or DL-lactic acid, or a mixture of two lactic acids. The D(-) lactic acid cannot be metabolized by humans and is not suggested for newborns and young children. The metabolic pathways of carbohydrates by LAB can be divided into two groups; (i) homofermentative (ii) heterofermentative (Figure 1). The homofermentative pathway converts sugars to the major lactic acid by Embden-Meyerhof pathway. Meanwhile, the heterofermentative bacteria produce not only

lactic acid but also ethanol, acetic acid, and CO_2 by the hexose monophosphate or pentose pathway (Axelsson, 2004; Caplice & Fitzgerald, 1999; Khalid, 2011).

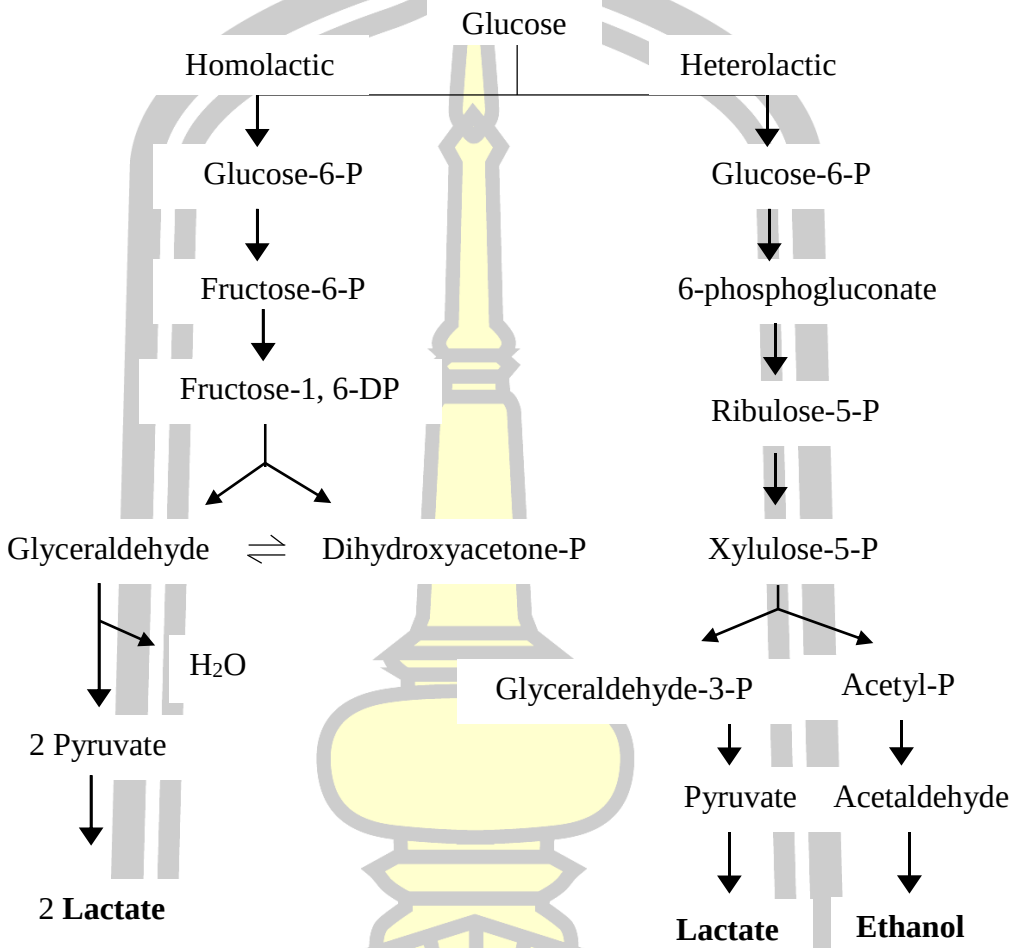


Figure 1 Fermentative pathway of glucose in lactic acid bacteria

Source: Caplice and Fitzgerald (1999)

2.5 Antimicrobial mechanism of lactic acid bacteria

LAB commonly used for food biopreservation are capable of producing organic acids, hydrogen peroxide, carbon dioxide, diacetyl, bacteriocin and reuterin (Ammor, Tauveron, Dufour, & Chevallier, 2006; Caplice & Fitzgerald, 1999).

2.5.1 Organic acids

LAB can produce organic acid such as lactic acid, acetic acid and propionic acid that possess an antimicrobial activity in fermented foods. The organic

acids have positive effects such as reduction of pH to a certain level for inhibition of putrefactive (e.g. *Clostridia* and *Pseudomonas*), pathogenic (e.g. *Samonellae* and *Listeria* spp.) and toxicogenic bacteria (*Staphylococcus aureus, Bacillus cereus, Clostridium botulinum*) in foods (W. Holzapfel, Geisen, & Schillinger, 1995). The organic acids help reduce the pH, due to the undissociated form of the molecules. The undissociated state is the active form which inhibits the pathogenic bacteria in foods. The undissociated acid's lipophillic presence can diffuse through the membrane causing damage to the electrochemical proton gradient, or destroy the cell membrane permeability responsible for exchanging the substrate transport system (Ammor *et al*., 2006).

2.5.2 Hydrogen peroxide (H_2O_2)

LAB cannot produce catalase enzyme to break down the H2 O2 produced in the presence of oxygen. Accumulation of H2O2 can act against some microorganisms. LAB can produce H2 O2 through fermentation in the presence of oxygen, and results in the production of flavoprotein oxidases or nicotinamide adenine dinucleotide (NADH) peroxidase. The H2O2 acts by denaturing enzyme due to the oxidation of sulfhydryl group which in turn results in the peroxidation of membrane lipids therefore increasing membrane permeability. H2O2 may activate the lactoperoxidase system in fresh milk by the formation of hypothicyanite and other antimicrobial products that inhibit bacteria such as Pseudomonas species and Staphylococcus aureus (W. Holzapfel et al., 1995). **2.5.3 Carbon dioxide**

Carbon dioxide is produced by LAB in a heterofermentative manner, that creates an anaerobic environment which inhibits enzymatic decarboxylation. Accumulation of CO_2 in the membrane lipid bilayer causes a dysfunction in permeability. The use of MAP (Modified Atmosphere Packaging) containing moderate to high level of CO_2 has an impact on food spoilage microorganism, especially gram-negative psychrotrophic bacteria. CO_2 affects cell membranes and can reduce internal and external pH (Caplice & Fitzgerald, 1999).

2.5.4 Diacetyl

Diacetyl is produced by some LAB; *Lactococcus, Leuconostoc* and *Pediococcus* spp. during citrate fermentation. It is an aromatic component. The

diacetyl predominantly impacts the inhibition of the growth of gram-negative bacteria, yeasts, and molds when compared to gram-positive bacteria (Caplice & Fitzgerald, 1999; W. Holzapfel *et al*., 1995; Piard & Desmazeaud, 1991). The mode of action entails interference with the arginine utilization. The concentration 200 µg/ml inhibits yeast and gram-negative bacteria growth at pH 5.5 (Jay, 1982). (Olasupo, Fitzgerald, Gasson, & Narbad, 2003) showed that the MICs (Minimum Inhibitory Concentrations) of diacetyl against *E. coli* and *S. typhimurium* are 12.5 and 7.5 $mmol^{-1}$, respectively.

2.5.5 Bacteriocins

The bacteriocins are peptide complex which are ribosomally synthesized from LAB. Bacteriocins are effective in inhibiting the growth of closely related bacteria and active against gram-positive. The action of bacteriocin on the cytoplasmic membrane is by the disruption of the proton motive force through the formation of pore in the phospholipids bilayer (Ammor *et al*., 2006; Caplice & Fitzgerald, 1999). Bacteriocin produced from LAB can be used as a starter culture for fermented foods in order to improve safety and quality (Callewaert, Hugas, & De Vuyst, 2000; Caplice & Fitzgerald, 1999; Karovičová & Kohajdová, 2005). Bacteriocin can inhibit the growth of pathogen such as *Listeria*, *Clostridium*, *Staphylococcus*, *Bacillus* spp. and *Enterococcus* spp. (Soomro, Masud, & Anwaar, 2002). Campos, Rodríguez, Calo-Mata, Prado, and Barros-Velázquez (2006) showed that bacteriocin from LAB isolated from turbot were able to prevent growth of *Listeria monocytogenese* and *Staphylococcus aureuse* and can be used as biopreservatives in fermented foods. Similarly, Mataragas, Drosinos, and Metaxopoulos (2003) showed that bacteriocin was produced from *Leuconostoc mesenteroides* L124 and *Lactobacillus carvatus* L442 against *L. monocytogenes* in meat. Huang *et al*. (2009) studied on bacteriocin produced by *Pediococcus pentosaceus* 05-10 isolated from a traditionally fermented vegetable product (Sichuan pickle) , and found that Pediocin 05-10 was able to reduce the number of *L. monocytogenes* 54002 in pork ham. Table 5 provides examples of bacteriocin produced by LAB isolated from food sources (Cleveland, Montville, Nes, & Chikindas, 2001).

2.5.6 Reuterin

Reuterin or 3-hydroxypropionaldehyde (Figure 2) produced from *Lactobacillus reuteri* during stationary phase from a mixture of glucose and glycerol or glyceraldehyde. It displayed broad-spectrum antimicrobial activity by inhibition of ribonucleotide reductase (Caplice & Fitzgerald, 1999; W. Holzapfel *et al*., 1995). It inhibits gram-negative and positive bacteria, yeasts and fungi (Helander, von Wright, & Mattila-Sandholm, 1997).

Figure 2 Structure of Reuterin

Source: Ulmer and Zeng (2007)

Table 5 Examples of bacteriocin produced by LAB isolated from foods

Source	Strain	Active against	References
Commercial probiotic product	*Streptococcus* sp. CNCM I-841	*Clostridium* sp., *L. monocytogenenes*	Gomez, Cosson, and Deschamps (1997)
Vegetable	*Enterococcus mundtii*	*L. monocytogenes*, *C. botulinum*	Bennik, Vanloo, Brasseur, Gorris, and Smid (1998)
Radish	*Lac. lactis subsp. Cremoris* R	*Clostridium*, *Staphylococcus*, *Listeria*, and *Leuconostoc* spp.	Bacillus (1998)
Bean-sprouts	*Lac. lactis* subsp. *lactis* (NisZ)	*L. monocytogenes*	Cai, Ng, and Farber (1997)
Dry sausage	*Lb. plantarum* UG1	*L. monocytogenes*, *Bacillus cereus*, *C. perfringens*, *C. sporogenes*	Enan, El-Essawy, Uyttendaele, and Debevere (1996)
Fermented sausage	*Lb. plantarum* SA6	*Lactobacillus* spp.	Enan *et al*. (1996)
Salad	*Lb. plantarum* BF905	*L. monocytogenes*, *Lb. sake*	Toit (1998)
Sour doughs	*Lb. bavaricus* (bavA)	*L. monocytogenes*	Larsen, Vogensen, and Josephsen (1993)
Whey	*Ent. faecalis* 226	*L. monocytogenes*	Villani *et al*. (1993)
Kimchi	*Lactococcus lactis* subsp. *lactis* A164	*S. aureus*, *L. monocytogenes*, *S. typhymurium*	M. Choi and Park (2000)

Table 5 Examples of bacteriocin produced by LAB isolated from foods (continued)

Source	Strain	Active against	References
Nham	*Lactococcus lactis* WNC20	*L. monocytogenes, Cl. perfrigens, B. cereus, S. aureus*	Noonpakdee, Santivarangkna, Jumriangrit, Sonomoto, and Panyim (2003)
Pla-Som	*Weissella cibaria* 110	Some gram-positive bacteria	Srionnual, Yanagida, Lin, Hsiao, and Chen (2007)
Chinese fermented cabbage	*Lactobacillus sake* C2	*S. aureus , E. coli*	Gao, Jia, Gao, and Tan (2010)
Tenerife cheese	*Lactobacillus plantarum* TF711	*B. cereus, Cl. sporogenes, S. aureus*	Hernandez, Cardell, and Zarate (2005)
Chinese fermented radish	*Lactobacillus sakei* LSJ618	*L. monocytogenes , S. aureus, Sacina sp., Micrococcus luteus, E. coli*	Jiang *et al*. (2012)
Bean-sprouts	*Lac. lactis* subsp. *lactis* (NisZ)	*L. monocytogenes*	Cai *et al*. (1997)
French mold-ripened Soft cheese	*Carnobacterium piscicola* CP5	*Carnobacterium, Listeria,* and *Enterococcus* spp.	Herbin *et al*. (1997)
Irish kefir grain	*Lac. lactis* DP3147	*Clostridium, Enterococcus, Listeria, Leuconostoc* spp.	Ryan, Rea, Hill, and Ross (1996)

2.6 Application of LAB starter cultures in food fermentation

2.6.1 Food preservative and safety

Chemical food additives such as nitrite, sulfite, propionic acid, sorbic acid, and benzoic acid are commonly applied in food preservation. The antimicrobial property from LAB is an alternative in food preservation. LAB can produce antimicrobials such as organic acids (lactic acid, acetic acid, formic acid), carbon dioxide, hydrogen peroxide, diacetyl, ethanol, bacteriocins, reuterin. Their occurrence may act against the growth of deleterious bacteria (Liu, Han, & Zhou, 2011). Sánchez, Rejano, Montaño, and de Castro (2001) studied the usage of *Lactobacilli* for Spanish-style green olive fermentation, the treatment with LAB inoculation indicated that the population of viable lactobacilli was higher than the uninoculated starter culture and inoculation reduced the population of *Enterobacteriaceae*, which is responsible for spoilage. In the sauerkraut production using *Lactobacillus plantarum* L4 and *Leuconostoc mesenteroides* LMG 7954 as starter culture indicated that the starter culture was responsible for rapidly decreasing pH and can inhibit the food-spoiling bacteria (Beganović *et al*., 2011). Acetic acid contributes to the aroma and inhibits mold in sourdough. Bacteriocin produced by LAB was used as an alternative to potassium nitrate to prevent the spoilage of cheese by *Clostridia* (Leroy & De Vuyst, 2004).

2.6.2 Improving organoleptic attributes

Fermentation can improve organoleptic properties of food. During fermentation, LAB can produce flavor metabolite such as diacetyl and organic acid which improve the taste of fermented products and the organic acids can interact with other substances (alcohols and aldehydes) resulting in production of additional flavor compounds (Bourdichon *et al*., 2012; Liu *et al*., 2011). The cabbage fermented with *Leuconostoc mesenteroides*, displays a firm texture and reduces off-flavors in the product (Johanningsmeier, McFeeters, Fleming, & Thompson, 2007).

2.6.3 Enriching nutrients

Fermentation can increase the nutritional value of the fermented product by increasing digestibility and removing toxic components of foods. The functions of LAB for improving the nutritional value involve: (*i*) The lactic acid from LAB can

increase the utilization ratios of calcium, phosphorus, and iron, in addition promotes adsorption of iron and vitamin D; (*ii*) the lactase can degrade lactose into galactose. The galactose is a component of cerebroside that can stimulate the growth of newborn's brain; (*iii*) proteinases from LAB can degrade casein into small protein molecules, which are easy to digest; and (*iv*) the fat of fermented dairy products with a globular texture is easy to digest and lipid breaks down, resulting in increase of non-esterified fatty acid content (Liu *et al*., 2011).

2.6.4 Increasing health benefits

Some LAB can colonize the guts of humans and animals and may be of benefit to the digestive system. They have been recommended to improve microcirculation in the gastrointestinal tract, increase immune function, control serum cholesterol levels (Shehata, El Sohaimy, El-Sahn, & Youssef, 2016; Wang *et al*., 2014), reduce intestinal infections and remove deleterious substances in consumer's body (Liu *et al*., 2011; Ranadheera, Baines, & Adams, 2010). *Weissella koreensis* FK121 isolated from fermented koozh indicated a cholesterol-reducing potential (Anandharaj *et al*., 2015). LAB such as *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophillus* found in yoghurt products can produce folate and the usage of these bacteria can result in increased folate content in dairy product (Crittenden, Martinez, & Playne, 2003; M. Lin & Young, 2000). According to Hunaefi, Akumo, and Smetanska (2013) red cabbages in natural fermentation, inoculated with *Lactobacillus plantarum* ATCC 8014 and inoculated with *Lactobacillus acidophilus* NCFM showed increased antioxidant activity with *Lb. plantarum* ATCC 8014 inoculation exhibiting highest antioxidant activity.

2.7 Selection criteria for lactic acid bacteria to be used as probiotic starter cultures

LAB have long been used as starter cultures in the production of fermented products. The functional starter cultures may result in an improvement in the fermentation process and improve quality of the end product. The selection of LAB strains used as starter cultures are chosen base on the following criteria: adapts easily to the raw material and process, develops enhanced organoleptic properties, extends shelf life, reduces the processing time and energy during the production, rapidly

accelerated metabolic activities (acidification or alcohol production), inhibits pathogenic microorganism, possess probiotic, non-pathogenic, and non-toxigenic properties (Corsetti *et al*., 2001; W. Holzapfel, 2002). According to M.-E. Lee *et al*. (2015) the use of starter culture in Kimchi fermentation enabled adaptability to the unique environment of fermentation such as low temperature, low pH, and the presence of NaCl. Table 6 indicated the characteristics of starter cultures and its effects on Kimchi fermentation. Nilchian, Sharifan, Rahimi, and MAZID (2016) studied fermentation of cucumber by using a starter culture which included *Lactobacillus plantarum*, *L. bulgaricus* and *S. thermophiles*, after inoculation with starter culture decreased pH during fermentation and high titratable acidity than the traditional process were observed which influences the spoilage by pathogenic bacteria and improves the safety of the product. Moreover, the starter culture contributes to the aroma and flavor of the fermented products. Karovičová and Kohajdová (2005) carried out studies of selected *Lactobacillus* strains on the fermentation of vegetable juice, in which the criteria used for strains selection were the rate of the pH decrease, organic acid production, nitrate and nitrite reduction and low content of biogenic amine. Biogenic amines are non-volatile low molecular weight nitrogenous organic bases, derived through decarboxylation of corresponding amino acids. They can be formed and degraded during the metabolism by human, animals, plants and microorganisms. The responsible enzymes are amino acid decarboxylases, which are widely present during spoilage by food microorganisms, i.e. naturally occurring and/or artificially added lactic acid bacteria (LAB) involved in fermentation in foods (Alvarez & Moreno-Arribas, 2014). The foods containing biogenic amine are responsible food toxicities. The consumption of foods containing a high concentration of these detrimental bacteria may cause flushes, headaches, nausea, cardiac palpitation, and fluctuations in blood pressure (Ladero, Calles-Enríquez, Fernández, & A Alvarez, 2010). If the concentration of biogenic amine is higher than 1000 mg/kg, it has adverse effects on consumers (Alvarez & Moreno-Arribas, 2014). The genera of *Enterobacteriaceae* and *Bacillaceae* as well as species of *Lactobacillus*, *Pediococcus* and *Streptococcus* are reported to exhibit decarboxylating of one or more amino acids. The major amines found in higher concentration in foods are histamine, tyramine, putrescine and cadaverine. The

biogenic amines found in fermented vegetables such as ethanolamine, putrescine, cadaverine, permidine, pheylethylamine, tyramine and histamine (Buckenhüskes, 1993). Table 7 summarizes the main criteria for selection of the starter culture for vegetable and fruit fermentation.

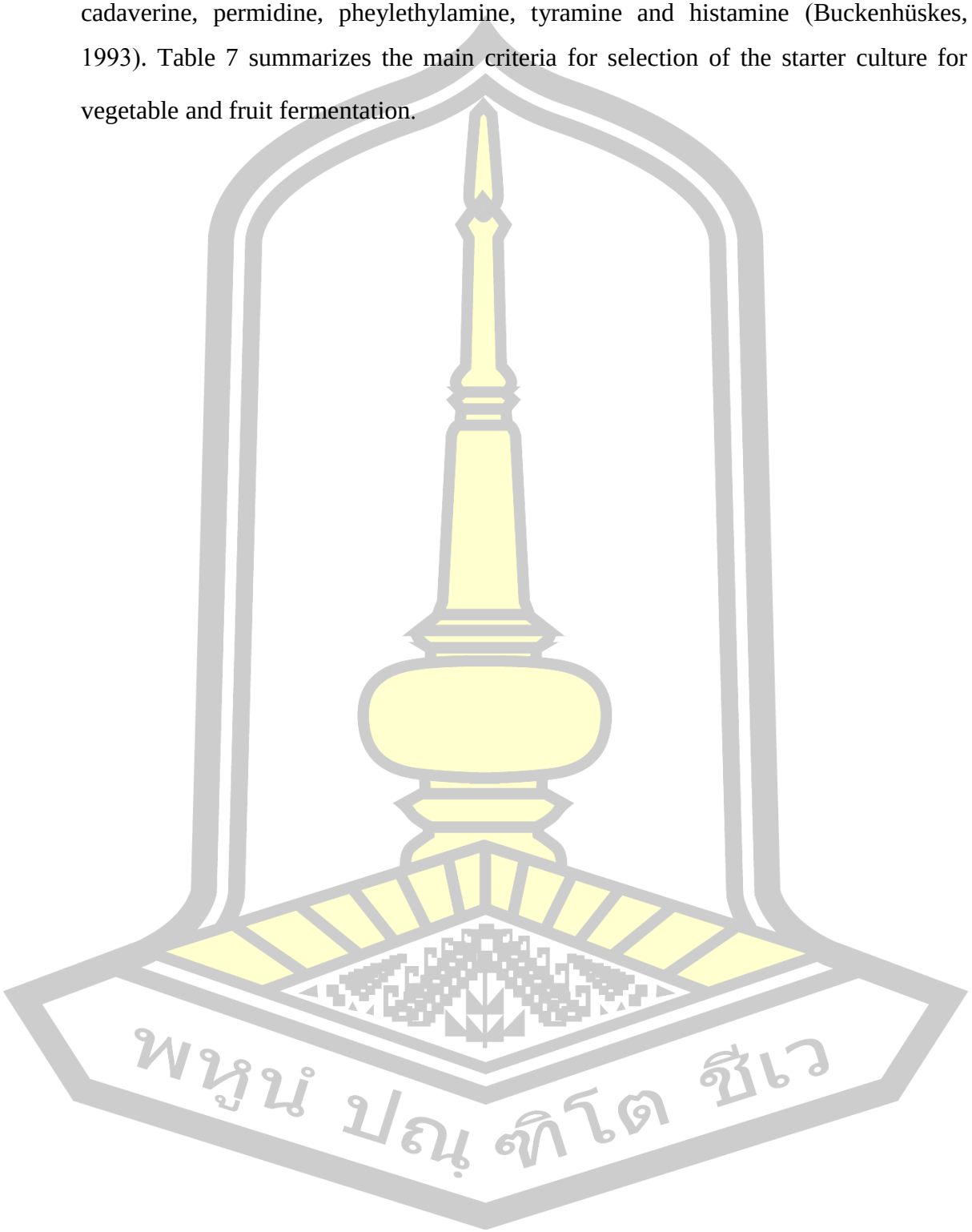


*Table **6*** The characteristics of starter cultures and effects on Kimchi fermentation

Starter culture	Characteristics	Effects on Kimchi	References
Leu.citreum IH22	Predominant lactic acid bacteria involved in kimchi fermentation	Maintained Kimchi quality for prolonged periods	I.-K. Choi *et al*. (2003)
Lb. plantarum NO1, *P. pentosaceus*, *Lb. plantarum* AF1	Tolerance to acid and bile	Improvement of functionality	Ryu and Chang (2013)
Leu. citreum GJ7	Production of bacteriocin	Prevented over-ripening and extended shelf-life	J. Y. Chang and Chang (2010)
Lb. plantarum PL62	Production of conjugated linoleic acids with anticancer and anti-obesity activities	Improvement of functionality	K. Lee and Lee (2010)
Leu. mesenteroides strain B1	Production of mannitol	Shortened the time to reach optimal ripened state	Jung *et al*. (2012)
Leu. mesenteriodes LK93	Resistance to acid and bile salts; antimicrobial and antifungal activities	Inhibition of the growth of film-forming yeast	Park *et al*. (2013)
Leu. citreum KACC91035	High dextransucrase activity	Improvement of isomaltooligosaccharide production	I.-K. Choi *et al*. (2003)

Table 7 The main criteria for selection of the starter culture in vegetable and fruit fermentation

Criteria	Metabolic traits
Pro-technological	Growth rate
	Acidification rate
	Salt tolerance
	Growth at low value of pH
	Tolerance to low value of pH
	Growth at low temperature
	Completeness of fermentation
	Malolactic fermentatiom
	Tolerance to phenol
	Synthesis of hydrogen peroxide
	Pectinolytic activity
Sensory	Hetero-fermentative metabolism
	Synthesis of aroma compounds or their precursors
Nutritional	Synthesis of exo-polysaccharides
	Synthesis of biogenic compound
	Increase of the antioxidant activity
	Synthesis of biogenic amine

Source: Di Cagno, Coda, De Angelis, and Gobbetti (2013)

2.8 Probiotics

Probiotics are live microorganisms when consumed in appropriate amounts confer health benefit to the host (FAO/WHO, 2002). The microorganism used in probiotic products include, *Lactobacillus*, *Bifidobacterium*, *Streptococcus*, *Enterococcus*, *Pediococcus*, *Propioniobacterium*, and *Saccharomyces*. The list of microorganisms used in dairy, pharmaceutical probiotic product is presented in Table 8. Typically, probiotic LAB are used for the production of functional foods and added to fermented foods. The probiotics are used because of the historical belief that these bacteria are required members of the intestinal micro flora. Traditionally, probiotics

have been added to yoghurt and other fermented dairy products such as cheese and ice-cream (Akın, Akın, & Kırmacı, 2007; Ranadheera *et al*., 2010), but certain people cannot consume the dairy product because of lactose intolerance and high cholesterol content and these are some of the drawbacks of dairy products. Table 9 shows traditional fermented food from several raw materials, which could help consumers with lactose intolerance and cholesterol restrictions (Rivera-Espinoza & Gallardo-Navarro, 2010).

*Table **8*** Probiotic bacteria in dairy product or pharmaceutical probiotic product

Genera	Species
Lactobacillus	*acidophilus*
	delbrueckii subsp. *bulgaricus*
	casei
	crispatus
	johnsonii
	lactis
	paracasei
	fermentum
	plantarum
	rhamnosus
	reuteri
Bifidobacterium	*adolescentis*
	bifidum
	breve
	essensis
	onfansis
	lactis
	longum
Enterococcus	*faecalis*
	faecium
Propioniobacterium	*freudenreichii*
Pediococcus	*acidilactici*
Streptococcus	*thermophilus*
Saccharomyces	*boulardii*

Source: Champagne, Gardner, and Roy (2005)

*Table **9*** Microorganisms used in probiotic traditional fermented products

Product	Probiotic microorganism	Substrates
Agbelina	*Lb. plantarum. Lb. brevis, Lb. fermentum, Leuc. mesenteroides*	Cassava
Boza	*Lb. plantarum. Lb. brevis, Lb. rhamnosus, Lb. fermentum, Leuc. mesenteroides* subsp. *dextranium*	Cereal
Dosa	*Leuc. mesenteroides, Lb. fermentum*	Rice and Bengal gram
Idi	*Leuc. mesenteroides*	Cereal, legume
Kenkey	*Lb. casei, Lb. lactis, Lb. plantarum, Lb. brevis, Lb. acidophilus, Lb. fermentum, Lb. casei*	Maize
Kimchi	*Lb. plantarum, Lb. curvatus, Lb. brevis, Lb. sake, Leuc. mesenteroides*	Vegetable
Kisra	*Lactobacillus* sp., *Lb. brevis*	Sorghum
Koko	*Lb. fermentum, Lb. salivarius*	Millet
Mahewu	*Lb. bulgaricus, Lb. brevis*	Maize
Mawe	*Lb. fermentum, Lb. brevis, Lb. salivarius*	Maize
Ngari	*Lactococcus lactis* subsp. *cremoris, Lactococcus plantarum, Enterococcus faecium, Lb. fructosus, Lb. amylophilus, Lb. coryniformis* subsp. *torquens*, and *Lb. plantarum*	Fish
Ogi	*Lb. plantarum, Lb. fermentum, Leuc. mesenteroides*	Maize
Sauerkraut	*Leuc. mesenteroides, Lactococcus lactis*	Cabbage

Source: Rivera-Espinoza and Gallardo-Navarro (2010)

2.9 Beneficial health effects and therapeutic value of probiotic

Probiotics hold potential for the prevention of disease in humans such as treatment of the gastrointestinal, respiratory and urogenital tracts diseases. Probiotics may provide an optimal balance and increase intestinal flora in the body, its ability to protect against infection by pathogens and maintain the host's well-being makes it an advantageous addition (Fooks & Gibson, 2002). Moreover, the maintenance of normal intestinal gut can enhance the immune system, reduce of lactose intolerance,

reduce serum cholesterol level and blood pressure, anti-carcinogenic activity, and improved utilization of nutrients and nutritional value of food. Probiotics have been used for therapeutic purposes such as prevention of urogenital diseases (candida vaginitis), alleviation of constipation, protection against traveler's diarrhea, prevention of infantile diarrhea, reduction of antibody-induced diarrhea, control of inflammatory bowel disease and irritable bowel syndrome, reduction of hypercholesterolemia, protection against colon and bladder cancer, prevention of osteoporosis and prevention of food allergy and atopic disease (Ranadheera *et al*., 2010). In addition, Sanders (2003) lists the targets in human for efficacy of probiotic (Table 10)

*Table **10*** Endpoints in human subjects for probiotic studies

Target	Proposed Mechanism
Allergy (atopic ezcema, milk allergy, rheumatoid arthritis)	Translocation/barrier effect
Carcinogenicity	Alteration of populations, activities, or ability to adhere to teeth of the oral microflora
Carcinogencity, mutagenicity, tumor	Mutagen absorption Immune stimulation Inhibition of carcinogen-producing intestinal micro flora
Cholesterol reduction	Deconjugation of bile acids
Diarrhea (antibiotic-associated, rotavirus, *C. difficile* colitis, travelers, community acquired)	Competitive exclusion Translocation/ barrier effect
Endotoxemia associated with alcoholic liver disease	Inhibition of endotoxin-producing intestinal micro flora
Helicobacter pylori	Anti-pathogenic activity

*Table **10*** Endpoints in human subjects for probiotic studies (continued)

Target	Proposed Mechanism
Kidney stones	Alteration of gut flora influencing oxalate degradation
Lactose intolerance	Delivery of microbial lactase to small intestine
Small bowel bacterial overgrowth	Antimicrobial activity, competitive exclusion
Vaginosis, urinary tract infections	Anti-pathogenic activity, competitive exclusion
Hypertension	Cell components or fermentation-derived peptides acting as ACE inhibitors
Immunomodulation (immune status, vaccine response)	Interaction with immune cell or cell receptors leading to increase phagocytic activity of white blood cells, increased serum IgA after antigen exposure, increased proliferation of intraepithelial lymphocytes
Irritable bowel syndrome; general gastrointestinal tract symptoms (constipation, non-pathogen-induce diarrhea, bloating, gas, cramping, gut-cause halitosis)	Alteration of population or activities of intestinal microflora
Inflammatory bowel diseases, ulcerative colitis, Crohn's pouchitis	Down-regulation of inflammatory

Source: Sanders (2003)

2.10 Selecting probiotic strain: important aspect

The foundation for the selection of probiotic bacteria such as safety, functional and technological features is demonstrated in Figure 3 (Saarela, Mogensen, Fondén, Mättö, & Mattila-Sandholm, 2000)

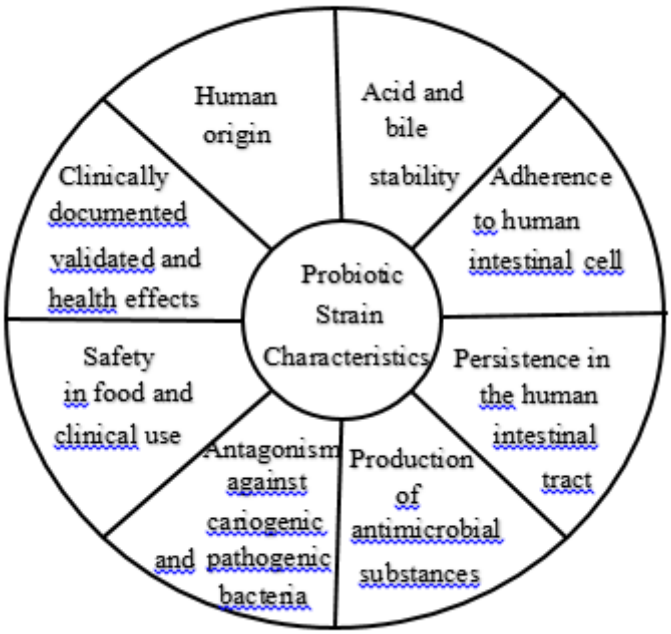


Figure 3 The foundation for the selection of probiotic bacteria
Source: Saarela *et al*. (2000)

The safety features are as follows:

1. Strains for human use are preferably of human origin.
2. They are isolated from healthy human GI-tract.
3. They have a history of being non-pathogenic.
4. They have no history of association with diseases such as infective endocarditis or GI-disorder.
5. They do not deconjugate bile salts.
6. They do not carry transmissible antibiotic resistance genes.

The functional requirements of probiotics should be established by using in vitro method. The probiotic strain must possess the following characteristics:

They can tolerance to human gastric juice.

1. They can tolerance to bile salts which important property for survival in small intestine.
2. They can adherence to epithelial cell and survival in human GI-tract.
3. They can enhance immune system.
4. They should have antagonistic activity such as inhibition of pathogens such as *Helicobacter pylori*, *Samonella* sp., *Listeria monocytogenese* and *Clostridium difficide*
5. They should have antimutagenic and anticarcinogenic properties.

The technological features have to be considered in probiotic selection. These include in terms:

1. Good sensory properties
2. Phage resistance
3. Viability during processing
4. Stability in the product and during storage

Morelli (2000) recommended that probiotic properties must have the following traits in order to be further tested for human probiotic use: it must be of human origin and survive during gastric transit, it must possess a tolerance to bile salts and gut epithelial tissue. Similarly as Jakubczak, Stachelska, Świsłocka, and Lewandowski (2012b) mentioned, the probiotic bacteria should tolerate the conditions in the gastrointestinal tract. They must be resistant to acid, bile, enzyme, low level of oxygen. The resistance to bile acids confirms that the probiotic bacteria reach the intestinal tract as living cells.

Swain, Anandharaj, Ray, and Parveen Rani (2014) mentioned that *L. acidophilus, L. paracasei, L. plantarum, L. reuteri* and *L. salivarius* are widely used as probiotic bacteria. The characters of probiotic bacteria can be chosen from several criteria (Table 11) e.g. it can survive passage through the gastrointestinal tract (GIT), when reach its location, it survives in the GIT and it should enhance function in the

gut environment. The characteristics of probiotic such as tolerance to gastric juice of human and bile salts, adherence to epithelial surface, survival in the GIT of human, enhance immune system, antagonistic activity to intestinal pathogen, and the ability to maintain and modulate the intestinal microbiota should be considered.

*Table **11*** Basic characteristics of selection of a probiotic strains

Characteristics of potential probiotic
Acid and bile stability
Human origin
Production of antimicrobial substances
Adherence to human intestinal cells
Persistence in the human intestinal tract
Clinically validated and document health effect
Antagonism against enteric pathogen
Susceptible to antibiotics
Safety in food and clinical use

Source: Swain *et al*. (2014)

2.11 *In vitro* valuation of potential probiotic bacterial

The several research have recommended that the valuation of potential probiotic bacterial are base on tolerance to gastric acidity and bile salt, adhesion to gut epithelial tissue, ability to colonize the gastrointestinal tract. Therefore, bacterial strains were evaluated based on probiotic properties within these parameters under laboratory condition.

2.11.1 Resistance to gastric acidity

The properties of probiotic bacteria should consider are tolerance to gastric acid because the probiotic bacteria are taken along with fermented food. After the food is consumed, the probiotic bacteria pass through the stomach and encounters the gastric juice secrete into the stomach that cause reduction of the pH in the stomach. The digestion of food in the human stomach take approximately 3 hours. and the pH of the stomach varies from 2.5 to 3.5 and can inhibit the microbes (W. H. Holzapfel *et al*., 1998). Therefore, the probiotic bacteria must be resistance to gastric acid in the

stomach, after which they move through into intestine tract (Henriksson, Khaled, & Conway, 1999). Karasu *et al*. (2010), conducted studied on survival of *Lactobacillus plantarum* under gastrointestinal conditions when using pepsin 0.3% at pH 2.0 and 3.0 for 0 and 3 hours and resistance was determined by the presence of viable cell. It was found that *Lactobacillus plantarum* can survive at pH 3 and also after incubation for 3 hours. Similarity, Lapsiri, Nitisinprasert, and Wanchaitanawong (2011) studied on the gastrointestinal tract tolerance of *Lactobacillus plantarum* isolated from fermented vegetable by using pepsin 0.3% at pH 2.0 and viable cell counts within 3 hours, some strain show ability to tolerate gastric juice at pH 2.0 for 180 min.

2.11.2 Resistance to simulated intestinal tract

The properties of probiotic bacteria should consider are resistance to intestinal tract because when probiotic bacteria survival from stomach and then pass through intestinal tract. The pH of intestinal tract is about pH 7.0-8.0 (Edwin *et al*., 1984). Charteris, Kelly, Morelli, and Collins (1998) mentioned pancreatic juice about 0.7 liters is secreted into the small intestine each day having a pH of about 8.0 and a salt content of not less than 0.5%. Lapsiri *et al*. (2011) studied on the tolerance small intestinal juice with the bile salt (pH 8) of *Lactobacillus plantarum* and viable cell counts after 4 hours, all strains were tolerance in simulated intestinal juice and 3 strains (TISTR 2073, TISTR 2077 and TISTR 2081) were the survival rate 84.90, 89.96 and 89.31%, respectively. Charteris *et al*. (1998) studied on small intestinal tolerance of probiotic *Lactobacillus* and *Bifidobacterium* species by using pancreatic juice (pH 8) and NaCl (0.5%), *Lactobacillus* and *Bifidobacterium* species retained viability during simulated small intestinal juice.

2.11.3 Bile acid resistance

The evaluating properties of lactic acid bacteria as probiotics are its ability to resist the effects of bile salt (Y.-K. Lee & Salminen, 1995; Swain *et al*., 2014). Bile acid is synthesized from cholesterol by the liver and it is stored in the gall bladder. The bile acid can degrade lipid and absorb vitamins insoluble in water. The volume of bile acid synthesized in humans is about 500-700 ml/day and at a concentration 0.3% (Morelli, 2000). When the food passes through the duodenum, the bile acid is secreted and it inhibits the growth of microorganism such as *Escherichia coli* sp., *Klebsiella* sp., and *Enterococcus* sp. However, gram-positive bacteria are found to be more

sensitive than gram-negative bacteria (Dunne *et al*., 2001). According to Taranto, Perez-Martinez, and de Valdez (2006) who studied the effect of bile acid on the cell membrane of *Lactobacillus reuteri* CRL 1098, found that bile salt destroyed the lipid bilayer structure of the cell membrane and inhibited sugar transport into cell that cause cell death. Therefore, the probiotic bacteria should be tolerance to bile acid and the bile salt tolerance of LAB isolated from several fermented product must be determined by supplementing with bile salt and the growth record using viable cells count (Bao *et al*., 2010; Boke, Aslim, & Alp, 2010; Mahasneh *et al*., 2015; Morelli, 2000; Tulini, Winkelströter, & De Martinis, 2013; Vera-Pingitore *et al*., 2016). In the study, the *Leuconostoc lactis*, *Lactobacillis plantarum*, and *Lactobacillus rhamnosus* isolated from plant fermentation can tolerate 0.3% bile salt and shows a good of cell viability after 24 hours (Vera-Pingitore *et al*., 2016). Meanwhile, *Lactobacillus pentosus*, *Lactobacillus brevis*, and *Lactobacillus salivarius* isolated from traditional fermented products showed a high number of cells after 24 hours at 0.3% and 0.5% of bile salt (Mahasneh *et al.*, 2015).

2.11.4 Adherence properties

Adherence properties are the one of the most important criteria for selection of probiotic bacteria. Adhesion of probiotic to the intestinal surface and the colonization of human GI-tract is an important requirement for probiotic activity. The mechanism of adherence of probiotics and pathogen microorganism onto the intestinal surface is the same therefore competition is observed, this in turn causes the pathogenic microorganism's in ability to adhere and is therefore dispose from the GI-tract (Saarela *et al*., 2000). Moreover, the adhesion requires an interaction with the mucus surface leading to the contact with the gut related lymphoid tissue mediating local and systemic immune effect. The HT-29 and Caco-2 cells are human intestinal cell lines that have been used for determining the adhesion property (Beganović et al., 2014; H. Lee *et al*., 2011; K. W. Lee *et al*., 2016). In studies, the adhesion of *Leuconostoc mesenteroides* and *Lactobacillus plantarum* isolated from traditional Korean fermented vegetable showed that *Leuconeostoc mesenteroid* adhered to HT-29 cells better than *Lactobacillus plantarum* (K. W. Lee *et al*., 2016). Meanwhile, Tuomola and Salminen (1998) reported that the four most adhesive strains were *L. casei*, *L. acidophilus* 1, *L. rhamnosus* LC-705, and *Lactobacillus* GG, where *L. casei*

var. *rhamnosus* was the least adhesive strain to Caco-2 cultures, indicating that the adhesion property was strain-specific among *Lactobacillus* spp. Moreover, the pig intestinal mucin are used to adhesion capacity (Carasi *et al*., 2014; Li, Yue, Guan, & Qiao, 2008; Valeriano, Parungao-Balolong, & Kang, 2014). Carasi *et al*. (2014) carried out adhesion properties of *Enterococcus* strains isolated from kefir by using porcine mucin, the 13 different *E. durans* strains adhesion to mucin ranged from 5.42 to 6.31 $\log_{10}$ CFU/cm^2.

2.11.5 Antimicrobial activity

Antimicrobial activities are importance criteria for selection of probiotic strains which indicate antagonism between colonic flora and pathogenic bacteria. The antibacterial substances can inhibited pathogenic bacteria such as bacteriocin, low molecular weight metabolites; organic acid, fatty acids, hydrogen peroxide, and diacetyl organic acid, fatty acids, hydrogen peroxide, and diacetyl. Several research efforts have focused on bacteriocin activity showed that bacteriocin has an inhibitory effect only against closely related species and spore formers such as *Bacillus* or *Clostridium* (W. Holzapfel *et al*., 1995). However, low molecular weight metabolites may be more important since they exhibit a wide inhibitory spectrum against many pathogenic bacteria e.g. *Samonella*, *Escherichia coli*, *Clostridium*, and *Helicobacter*. In studies, the culture supernatant of *Lactobacillus caesei* ability to antagonize *Samonella typimurium*, *Bacillus cereus*, *Escherichia coli*, *Staphylococcus aureus*, and *Shigella dysenteriae* but this activity was attributed to organic acid and no specific compound was responsible for the inhibitory effect (V. Mishra & Prasad, 2005). The culture supernatant of human *Lb. acidophilus* strain LB decreased the number of *S. aureus*, *Lb. monocytogenes*, *S typhimurium*, *Shigella flexneri*, *E. coli*, *Klebsiella pneumonia*, *B. cereus*, *Pseudomonas aeruginosa* and *Enterobacter* spp. (Coconnier, Liévin, Bernet-Camard, Hudault, & Servin, 1997). The test of probiotic activities (antimicrobial properties) by using the cell-free supernatant of *Lb. casei* subsp. *rhamnosus* strain showed inhibition of human pathogenic bacteria, enterotoxigenic *E. coli*, enteropathogenic *E. coli*, *E. faecalis*, and *Cl. difficile*. The growth of all strains were inhibited (Forestier, De Champs, Vatoux, & Joly, 2001).

2.11.6 Hydrophobicity property

Hydrophobic properties of bacterial surfaces are a major determinant in the adhesion of bacteria. Hydrophobicity is likely due to a complex interplay between negatively-charged, positively-charge, hydrophobic and hydrophilic component on the surface of the bacteria (Abdulla, Abed, & Saeed, 2014). The hydrophobicity property is related to the adhesion ability to the host's intestinal mucus due to the high hydrophobicity shown by the bacteria strain (Karasu *et al*., 2010; Ouwehand, Kirjavainen, Grönlund, Isolauri, & Salminen, 1999; Ram & Chander, 2003). The hydrophobicity of bacteria cell is due to hexadecane which is a non-polar hydrocarbon. According to Karasu *et al*. (2010) studies on hydrophobicity property of *Lb. plantarum* by using hexadecane, found that the isolated *Lb. plantarum* strain showed hydrophobicity between 30% and 80%, *Lb. plantarum* 3 showed highest hydrophobicity of 80%. Klayraung *et al*. (2008) carried out cell surface hydrophobicity of *Lactobacilli* from Thai fermented food using hexadecane, the results revealed the highest was 68.7% and lowest hydrophobicity was 20.9%.

2.11.7 Other selection criteria for probiotic LAB

Mokoena, Mutanda, and Olaniran (2016) recommended that ideal probiotic LAB properties must include; resistance to antibiotic, antimutagenicity properties, rapid production of lactic acid, viability and stability in storage, ability to stimulate the host immune response, and the ability to influence metabolic activities such as vitamin production, cholesterol removal (Wang *et al*., 2014), and lactose utilization (Pundir, Rana, Kashyap, & Kaur, 2013). Moreover, Ji *et al*. (2013) studies on properties of *Lactobacillus plantarum* and *Leuconostoc citreum* isolated from Korean Kimchi and preliminary in vitro test for safety consideration showed antibiotic resistance, haemolysis and biogenic amine production. These indicated *Lactobacillus plantarum* had the safety and functionality of probiotic.

2.12 Fermented vegetables

The fermentation of vegetable material is an old preservative method. The advantage of fermented vegetable products includes: the high degree of hygienic safety caused by growth control of pathogenic bacteria, product can be promoted as natural or biological, production of flavor metabolite compound and inhibition of undesirable flavor compounds such as glucosinolates, using the energy less than the

other energy preservative method, easy management and storage without refrigeration, and an easy method for management of raw material before more processing (Maki, 2004). Most fermented vegetable products such as sauerkraut juice is produced by natural fermentation, which typically involves a host of microbial populations. The amounts of naturally occurring lactic acid bacteria (LAB) in fresh vegetables are very low. Lactic acid bacteria found on plant material are presented in Table 12

*Table **12*** LAB involved in plant materials

LAB in plant material
Lactobacillus brevis
Lactobacillus casei
Lactobacillus plantarum
Lactobacillus arabinosus
Lactobacillus buchneri
Lactobacillus fermentum
Leuconostoc mesenteroides
Pediococcus acidilactici
Pediococcus pentasaceus (formerly P. cerevisiae)
Enterococcus faecalis (formerly *Streptococcus faecalis*)
Enterococcus faecalis var. *liquefaciens*
Enterococcus faecalis (formerly *Streptococcus faecium*)
Lactococcus lactis (formerly *Streptococcus lactis*)

Source: Maki (2004)

Vegetables are good sources of water soluble vitamins such as vitamin C and B-complex, provitamin A, phytosterols, dietary fibers, minerals, and phytochemicals. Vegetables have low sugar content but rich in mineral and vitamins and have neutral pH and thus provide a natural medium for lactic acid fermentation (Swain *et al*., 2014). The Fermentation of vegetables can occur spontaneously by the natural lactic acid bacteria surface microflora, such as *Lactobacillus* spp., *Leuconostoc* spp., and *Pediococcus* spp. The raw material and microorganism involved in traditional

fermented vegetable are present in Table 13. The fermentation of vegetable is well suited to promoting health because its a source of probiotic bacteria. Kimchi is fermented vegetable. It is popular as a functional food because of its high content of vitamins, minerals, fibers and phytochemicals and probiotic properties (H. Lee *et al*., 2011). Probiotic *Lb. plantarum* strain L4 and *Leuconostoc mesenteroides* strain LMG 7954 were used in the fermentation of cabbage heads for improving functional value and decreasing NaCl level. The results indicated the fermented cabbage heads have functional value (probiotic properties) and can produce fermented cabbage head with lowered use of NaCl from 4.0% to 2.5% (Beganović *et al*., 2011). Some of the fermented fruits and vegetables contain phytochemicals such as flavonoids, lycopene, anthocyanin and glucosinolates. These phytochemicals have antioxidant activity and protect our cells against oxidative damage and reduce the risk of developing certain types of cancer (Kaur & Kapoor, 2001). According to Kaur and Kapoor (2001) studies on the antioxidant properties of white cabbage after heating and fermenting prove that fermentation processes and heat treatment improve the antioxidant activity of cabbage.

Table ***13*** Examples of traditional fermented vegetable of Asian nations

Fermented product	country	vegetable	microorganism	references
Soidon	India	Bamboo shoot	*L. brevis, L. fallax, L. lactis*	B. Tamang *et al.* (2008)
Suan-tsai	Taiwan	Chinese cabbage, cabbage, Mustard leaves	*P. pentosaceus, Tetragenococcus halophilus*	Y. S. Chen, Yanagida, and Hsu (2006); Lu, Peng, Cao, Tatsumi, and Li (2008)
Sauerkraut	International	Cabbage	*Leu. mesenteroides, L. plantarum, L. brevis, L. plantarum, L. rhamnosus*	Viander, Mäki, and Palva (2003); Z. Yang *et al.* (2010)
Pak-Gard-Dong	Thailand	Mustard leaf	*L. brevis, P. cerevisiae, L. plantarum*	S Tanasupawat and Komagata (1995)
Pak-sian-dong	Thailand	Leaves of Pak-sian	*L. brevis, P. cerevisiae, L. plantarum*	S Tanasupawat and Komagata (1995)
Gundruk	Nepal, India	Cabbage, radish, mustard, cauliflower	*Lactobacillus and Pediococcus* spp.	Dahal, Karki, Swamylingappa, Li, and Gu (2005)
Kimchi	Korea	Cabbage, radish, various vegetables	*Leuconostoc mesenteroides, L. brevis, L. plantarum, L. sakei*	J.-S. Lee *et al.* (2005)
Olive	Spain, Italy	Olive	*L. plantarum, L. brevis, L. pentosus,*	Argyri *et al.* (2013)

*Table **13*** Examples of traditional fermented vegetable of Asian nations (continued)

Fermented product	country	vegetable	microorganism	references
Paocai	China	Cabbage, celery, cucumber, and radish	*L. plantarum*, *L. brevis*, *L. pentosus*, *L. lactis*, *Leu. mesenteroides*, *L.fermentum*	Feng, Chen, Li, Nurgul, and Dong (2012); Yan, Xue, Tan, Zhang, and Chang (2008)
Sauerkraut	International	Cabbage	*Leu. mesenteroides*, *L. plantarum*, *L. brevis*, *L. plantarum*, *L. rhamnosus*	Viander *et al*. (2003); Z. Yang *et al*. (2010)

2.13 The research study on isolation lactic acid bacteria with probiotic properties in fermented vegetable product

H. Lee *et al*. (2011) studied isolation of probiotic lactic acid bacteria from Kimchi, it was found *Lactobacillus sakei* and *Lactobacillus plantarum*. These strains were studied on probiotic properties, all strains were survival in gastrointestinal tract, adherence to HT-29 cell. These strain showed antimicrobial properties against pathogenic bacteria.

J. P. Tamang *et al*. (2009) studied functional properties of LAB strains isolated from ethic fermented vegetables. LAB strains were *Lb. brevis*, *Lb. plantarum*, *Lb. curvatus*, *P. pentosaceus*, *P. acidilactici*, *Leuc. mesenteroides* subsp. *mesenteroides*, *Leuc. fallax*, *Leuc. citreum* and *E. durans*. LAB strains were evaluated for functional properties; production of biogenic amine, hydrophobicity and adherence to mucus HT29 MTX cells and antimicrobial activity. Most of the LAB strains showed antimicrobial activities against the bacteria indicator and not produce biogenic amine. Some strains of *Lb. plantarum* revealed more than 70%

hydrophobicity. Seven strains were able to adhere to mucus secreting HT29 MTX cells.

Ji *et al*. (2013) studied functional properties and safety of *Lactobacillus plantarum* strains and *Leuconostoc citreum* strain isolated from Kimchi; antibiotic resistance, haemolysis, biogenic amine production and survival in simulated gastrointestinal. All strains were susceptible to antibiotic (erythromycin, gentamicin, ampicillin, tetracycline, chloramphenicol, streptomycin, ciprofloxacin and benzylpenicillin). The results showed all strains not production of biogenic amine and no haemolytic activity. All strains were able to survive in simulated gastrointestinal.

Mahasneh *et al*. (2015) isolated LAB from fermented vegetable, the results showed *Lactobacillus plantarum*, *Lactobacillus pentosus*, *Lactobacillus brevis* and *Lactobacillus salivarius*. They were studied on probiotic properties, *Lactobacillus plantarum* were able to survive in acidity and intestinal condition. All isolates were able to inhibited the growth of pathogenic bacteria.

S.-M. Chang *et al*. (2013) studied on isolation and probiotic properties of LAB isolated from Taiwan traditional paocai. LAB isolates were identified as *Lactobacillus plantarum, Lactobacillus casei*. LAB strains were able to survive at pH 2.0 and 0.3% bile salt, showed antimicrobial activity inhibited pathogenic bacteria (*Bacillus cereus*, *Micrococcus luteus*, *Salmonella typhimurium* and *Staphylocccus aureus*). *Lactobacillus plantarum* E51 revealed the highest adherence properties.

Argyri *et al*. (2013) studied probiotic properties of LAB isolated from fermented olives, the results showed *Leuconostoc mesenteroides*, *Leuconostoc pseudomesenteroides*, *Lactobacillus plantarum*, *Latobacillus pentosus*, *Lactobacillus paraplantarum*. *Lactobacillus paracasei* subsp. *paracasei*. They were evaluated probiotic properties, the results exhibited *Lb. pentosus*, *Lb. plantarum* and *Lb. paracasei* subsp. *paracasei* were able to survive in low pH condition. The majority of LAB strains were survive to bile salt condition. All strains revealed resistance to vancomycin. *Lb. pentosus* E108, *Lb. plantarum* B282 and *Lactobacillus paracasei* subsp. *paracasei* E94 showed high adherence properties.

Ryu and Chang (2013) studied probiotic properties of LAB isolated from Kimchi; *Lactobacillus buchneri*, *Lactobacillus plantarum*, *Leuconostoc citreum*, *Leuconostoc mesenteroides*. *Pediococcus pentosaceus*. These strains were not haemolytic activity, showed no antibiotic resistance. Some strains of *Lactobacillus plantarum* and *Pediococcus pentosaceus* showed high tolerance to acid, bile condition, adherence to Caco-2 and HT-29 cell and inhibited pathogenic bacteria (*Staphylococcus aureus*, *E. coli* O157:H7, *Salmonella typhi*, and *Listeria monocytogenes*).

Lapsiri *et al*. (2011) studied on probiotic properties of *Lactobacillus plantarum* strains isolated from fermented vegetables. All strains were susceptible to chloramphenicol, rifampin and penicillin. Most strains showed antimicrobial properties inhibit the pathogenic bacteria. Seven strains were able to survive in acid and bile salt condition. *Lb. plantarum* TISTR 2075 had good probiotic properties and appropriate strain for food application.

Karasu *et al*. (2010) studied on probiotic properties of *Lactobacillus plantarum* strains isolated from traditionally produced fermented vegetables. All *Lb. plantarum* strains were able to survive low pH and high bile salt (7%). The antibiotic susceptibilities showed resistant to ampicillin, erythromycin, chloramphenicol and rifampin.

Mourad and Nour-Eddine (2006) studies probiotic properties of *Lactobacillus plantarum* strains from fermented olives, the results showed all strains were susceptible to penicillin G, ampicillin, vancomycin, chloramphenicol, clindamycin, rifampicin and ciprofloxacin. *Lb. plantarum* OL15 strain showed the highest survival rate (pH 2.5) and 2 strains; *Lb. plantarum* OL15 and OL16 showed the highest bile salt tolerance. All strains revealed no haemolytic activity.

2.14 Fermented Pak-Sian

Fermented Pak-Sain is a pickled leafy vegetable (*Cleome gynanda*). The preparation of fermented Pak-Sain is simple. The fresh vegetable is cleaned with water and the spread out and expose to the sun for water evaporation until the

vegetable is flaccid. The formula in fermentation include: 2-4% salt, 5% sugar. Sometime, to reduce bitter flavor, the leaves are soaked in water and salt overnight. It is then mixed with water, salt, sugar and keep in glass jar or container, fermentation for 3-5 day at room temperature in Figure 4. The pH of product is about 3.90 and the acidity is in 0.7-0.8% (Steinkraus, 2018). LAB involved during fermentation were *Lb. brevis*, *P. cerevisiae*, *Lb. plantarum*, *Lb. buchneri and Lb. fermentum* (Steinkraus, 2018; Swain *et al*., 2014).

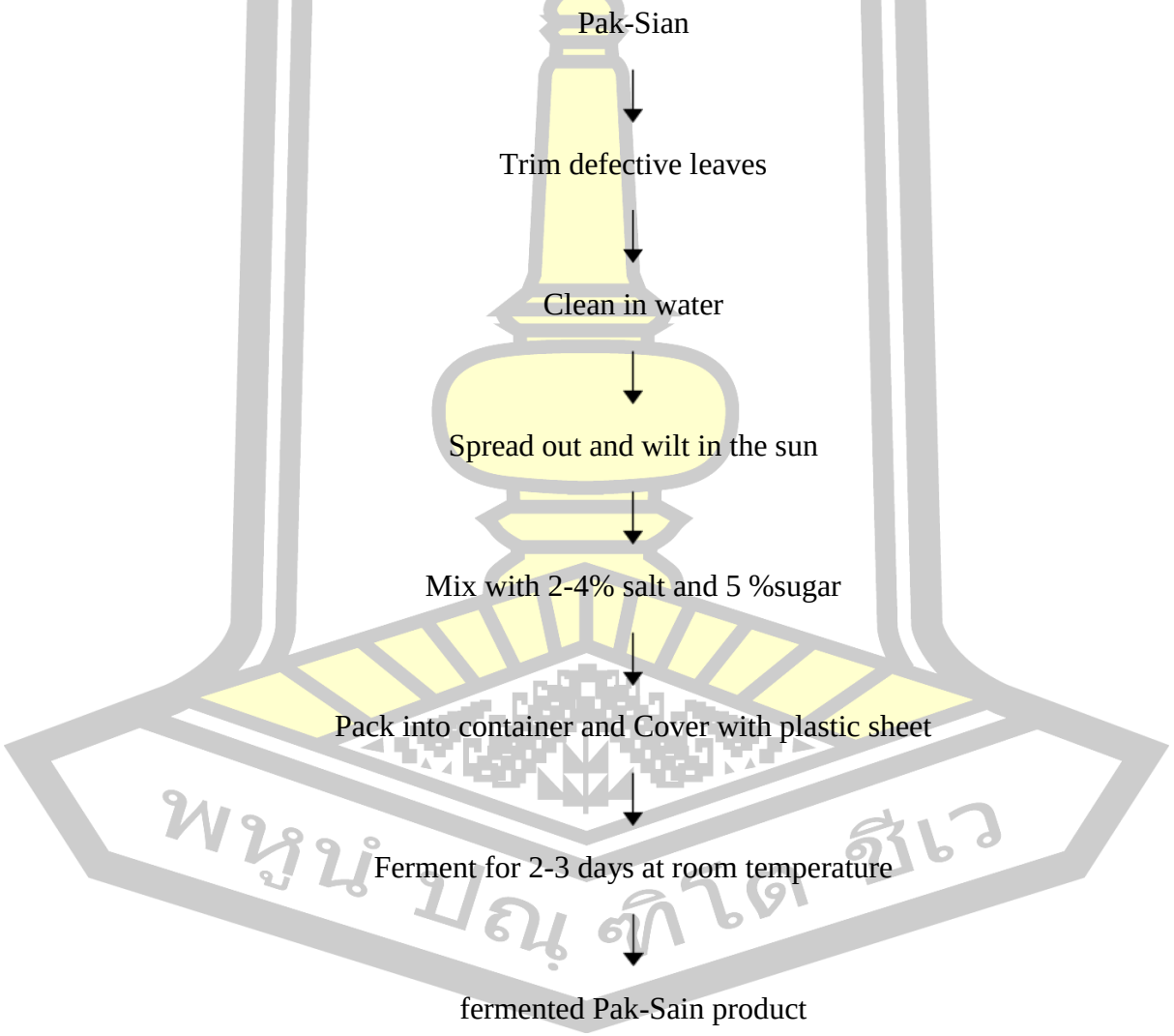


Figure ***4*** Flow chart of fermented Pak-Sain fermentation

Fermented Pak-Sian is a local fermented vegetable and widely consume in Thailand especially, the north-eastern and northern region. Fermented Pak-Sian is a lactic fermentation (Swain *et al*., 2014). LAB involved during fermentation were *Lb. brevis*, *P. cerevisiae*, *Lb. plantarum*, *Lb. buchneri* and *Lb. fermentum* (Steinkraus, 2018; Somboon Tanasupawat, 2009; S Tanasupawat & Komagata, 1995).

Somboon Tanasupawat *et al*. (1992) characterization and identification of LAB from several fermented foods in Thailand such as Pak-sian-dong by using phenotypical and genotypical technique, the results reveal that found *Lb*. *pentosus* F 11-1, *Lb*. *pentosus* F4-1, *Lb*. *pentosus* F 24-1, and *Lb*. *pentosus* F 18-1.

Somboon Tanasupawat and Daengsubha (1983) studies on *Pediococcus* sp. in fermented food in Thailand such as fermented fish, meat, vegetable and other material in Thailand by using morphological characteristics, cultural characteristics, production of acid from sugars, biochemical characteristic, and physiological characteristics, the results reveal that found 22 isolates were *Pediococcus pentosaceuse*, 2 isolates were *Pediococcus acidiococcus*, 26 isolates were *Pedicoccus halophilus*, 4 isolates were *Tetracoccus* sp. In this study, had investigated isolation bacteria from fermented Pak-Sian, it was found *P. pentosaceus*. The investigated *Pedicoccus* sp. due to it is important in souring and ripening of fermented foods.

CHAPTER III
RESEARCH METHODS

3.1 Isolation and Identification of LAB from fermented Pak-Sian product

3.1.1 Collection of fermented Pak-Sian product

In this study, about 600 g of the samples (300 g of solid portion+ 300 g of liquid portion) was collected from the local markets of the following 4 provinces: Kalasin, Sakon Nakhon, Maha Sarakham and Khon Kaen. The samples was collected and kept at 4 °C in a refrigerator until further chemical and microbiological analysis (the maintenance of sample should not exceed 24 hours).

3.1.2 Chemical analysis

The pH of the sample was measured by a pH meter. The titratable acidity (% lactic acid) was measured by titration against a standard solution of 0.1 M NaOH using phenolphthalein as an indicator (Appendices A). The NaCl level was determined by titration against 0.1 M $AgNO_3$ and 5 % K_2CrO_4 as an indicator according to AOAC (2000) (Appendices A). Triplicates of samples was used in this experiment and analysis was done by calculating mean and standard deviations.

3.1.3 Microbiological analysis

The microbiological analysis of samples was done by viable cell counts of LAB using serial dilution on MRS agar supplemented with 0.05% bromocresolpurple (BCP) indicator incubated at 37 °C for 48 hours (Beganović *et al*., 2011).

3.2 Isolation of LAB from fermented Pak-Sian

The samples were collected from the local markets of the following 4 provinces: Kalasin, Sakon Nakhon, Maha Sarakham and Khon Kaen. The samples were collected and kept at 4 °C in a refrigerator until further study. The sample (10 g) was dissolved in 90 ml of 0.85% NaCl solution to make a serial dilution. The 0.1ml was isolated using spread plate method using De Man Rogosa and Sharpe (MRS agar) added with Bromocresol purple (BCP) incubated at 37 °C for 24 h. The selected single colony that forms a yellow zone on BCP was purified by streaking on MRS agar (10-12 times) and morphology was tested using gram straining. Catalase test was

carried out by 5% H_2O_2. The LAB isolate must be gram-positive and catalase-negative (Vera-Pingitore *et al*., 2016; Xiong, Guan, Song, Hao, & Xie, 2012). LAB isolates were analyzed according to the whole cell-protein pattern. The 2 ml of LAB isolates were cultured in MRS broth for 24 h at 37 °C. Then, the cell suspension were centrifuged at 12,000 g for 5 min, the sample mixed with the sample buffer (0.5M Tris-HCl, 10% SDS, glycerol, 2-Mercaptoethanol and bromophenol), sonicated for 5 min, heated at 95 °C for 5 min and centrifuged at 12,000 g for 5 min, and the supernatant was used for SDS PAGE analysis. The condition of SDS-PAGE included 12.5% separating gel, 4% stacking gel, electrophores at 150V for 60 min. The gel was stained with Comassie Brilliant Blue R-250 for 15 min, destained with destaining solution until the gel background was clear. 1 ml overnight culture of isolated LAB was pelleted by centrifugation at 10,000 rpm for 2 min. The pellets were stored at -20 °C for further genomic DNA extraction. The genomic DNA was extracted by using bacterial genomic DNA extraction kit (Vivantis, Malaysia). The genomic DNA extraction was performed according to the kit instructions. The PCR conditions are according to (Luang-In & Deeseenthum, 2016). The reaction was carried out using the forward primer AmpF (5’- GAGAGTTTGATYCTGGCTCAG-3’ and the reverse primer AmpR (5’-AAGGAGGTGATCCARCCGCA-3’). The PCR cleanup was done using GF-1 PCR clean up kit (Vivantis, Malasia) and performed according to the instruction kit. The targeted 16s rDNA gene were sent to sequencing at 1st Base Co. Ltd (Malaysia). The identification was refined after the BLAST alignment of 16S rDNA sequences. The purified LAB isolate was maintained in MRS broth containing 20% of glycerol and stored at -20 °C for further experimentation.

3.3 Study on probiotic properties

3.3.1 Bile salt tolerance test

Bile salt was evaluated described modified according to K. W. Lee *et al*. (2016) and Vera-Pingitore *et al*. (2016). LAB were cultivated on MRS broth overnight and adjusted with 0.5 McFarland for preliminary use. The 200 µl of LAB was added to MRS broth including 0.3% oxall, and incubated for 24 h. The total viable count was determined after 24 h of incubation time by MRS agar and compared with the control culture (without bile salt), serial dilution was carried out using sterile

0.85% NaCl, incubation was carried out at 37°C for 48 h. The Survival rate (%) was calculated (Bao *et al*., 2010)

Survival rate (%) = (Log N/Log N_0) × 100

Log N represents the number of viable count after incubated for 24 h.

Log N_0 represents the initial viable count prior to exposure.

3.3.2 pH tolerance

LAB were evaluated pH tolerance by modified according to K.W. Lee *et al*. (2016); Tulini *et al*. (2013); Vera-Pingitore *et al*. (2016). LAB isolates were cultivated on MRS broth overnight (about 18 h) and adjusted with 0.5 McFarland for preliminary use. 200 µl of LAB was added to MRS broth (pH 2.5), and incubated for 180 min. The total viable count was determined after 24 h of incubation time using spread plate technique on MRS agar and comparing with the control culture (pH 7.2), serial dilution was carried out using sterile 0.85% NaCl, incubation was carried out at 37°C for 48 h**.** The Survival rate (%) was calculated.

Survival rate (%) = (Log N/Log N_0) × 100

Log N represents the number of viable count after incubated for 180 min.

Log N_0 represents the initial viable count prior to exposure.

3.3.3 Survival in simulated gastric and intestinal tract

Survival in simulated gastric and intestinal tract was studied according to modified K. W. Lee *et al*. (2016); Pieniz, Andreazza, Anghinoni, Camargo, and Brandelli (2014). LAB isolates were cultivated in MRS broth overnight. Suspension was centrifuged (12,000 g for 5 mins, at 4 °C), washed twice in phosphate buffer saline (pH 7) and re-suspended in 3 ml of 0.5% NaCl and adjusted to OD_{600} as 0.1. Sample LAB suspension (1 ml) was added to 9 mL of gastric solution (0.3% pepsin in sterile 0.5% (w/v) NaCl, pH 3.0), control and carried out before and after the viable count on MRS agar using spread plate technique after incubation for 3 h at 37°C. Sample LAB suspension (1 ml) was added to 9 ml of intestinal juice solution (0.1% w/v pancreatin) in sterile 0.5% w/v NaCl, pH 8.0) and the control (0.5% NaCl, pH 7) after an inoculation time of 4 h at 37 °C. The total viable LAB count was carried out on MRS agar and diluted with 0.85% NaCl using spread plate technique, at an

incubation time of 48 h at 37 °C. The total viable count is reported as log CFU/ml. The Survival rate (%) was calculated.

Survival rate (%) = (Log N/Log N_0) × 100

Log N represents the number of viable count after exposure.

Log N_0 represents the initial viable count prior to exposure.

3.3.4 Antibiotic susceptibility

Antibiotic susceptibility of LAB isolates were determined by the overlay diffusion method according to modification of Cebeci and Gürakan (2003). LAB isolates were cultivated in 5 ml MRS broth, overnight at 37 °C and adjusted to OD_{600} as 0.1 for preliminary use. Then, MRS agar were poured onto the plates and covered with 4 ml soft agar containing 200 µl of LAB culture. The plates were kept at room temperature for 1 h. Antibiotic discs were placed on the culture media and incubation was carried out at 37 °C for 24 h. The 6 antibiotics used are: ampicillin (10µg) and vancomycin (30µg) for inhibition of cell wall synthesis. Azythromycin (15µg), chloramphenicol (30µg) and streptomycin (10µg) for inhibition of protein synthesis and rifampicin (5µg) for inhibition of nucleic acid synthesis. Inhibition zones were reported in term of resistant (R), moderate susceptibility (M) and susceptible (S) (Charteris *et al*., 1998) (Appendices F). The diameters of inhibition zone were measured using the Vernier caliper.

3.3.5 Antimicrobial activity of LAB

The detection activity of LAB were carried out using agar spot method according to the modification of the technique used by Carasi *et al*. (2014). The LAB strains were cultivated in MRS broth at 37 °C and overnight. The 5 µl of LAB culture were spotted on MRS agar and incubated at 37 °C for 24 h. The bacteria indicators such as *E. coli, S. typhimurium, B. cereus* and *S. aureus* were inoculated in a nutrient broth and incubated overnight at 37 °C and the OD_{600} was adjusted to 0.1-0.12 about 10^8 CFU/ml. The bacterial indicators of 0.25 ml were inoculated into 7 ml of soft nutrient agar (0.7% agar) and poured onto MRS agar and incubated at 37 °C for 24 h. The antibacterial activity was determined by measuring the inhibition zone around the LAB spot. Inhibition zone was verified as the diameter of the zone (mm) around the colonies; + shown as diameter of inhibition zone less than 10 mm; ++ shown as

diameter of inhibition zone, 10 to 20; +++ shown as diameter of inhibition zone, above 20 mm.

3.3.6 Detection of biogenic amine production

The detection of biogenic amine production was carried out according to the modification of the technique used by modified according to H. Lee *et al*. (2011). The LAB isolates were cultivated in MRS broth by incubation overnight and the culture was normalized to 1.0 at OD_{600}. The LAB isolates were subcultured 5 times in MRS broth containing 0.1% of each precursor amino acid (L-Tyrosine disodium salt, L-Histidine monohydrochloride monohydrate, L-Ornithine monohydrochloride, and L-Lysine monohydrochloride) in order to promote the enzyme induction. After activation, the LAB isolates were streaked on decarboxylase medium containing 1% of each amino acid, bromocresol purple was used as pH indicator and without amino acid as control. The plates were incubated for 4 days at 37 °C. A positive result is indicated by a change of the medium color to purple in response of the indicator to pH increase. The pH change is related to more alkaline biogenic amine production from the amino acids initially included in the medium.

3.3.7 Haemolytic activity

Haemolytic activity was evaluated according to modified (Ji *et al*., 2013; Pieniz *et al*., 2014). The LAB strains were inoculated and cultured overnight in MRS broth at 37 °C and streaked on Columbia agar plates, consisting of 5% (v/v) sheep blood and incubated for 48 h at 37 °C. The culture plates were examined for β-haemolysis (clear zone around colonies), α- haemolysis (green-hued zone around colonies) and γ- haemolysis (none zone around colonies). The haemolytic activity was considered negative.

3.3.8 Adhesion capacity to pig intestinal mucin

The adhesion capacity was estimated according to the method described according Valeriano *et al*. (2014), 100 µl of the mucin solution (1 $mgml^{-1}$) in 10 mmol/ l HEPES-Hanks (HH) buffer was immobilized on 96-well polystyrene micro titer plate and incubated overnight at 4 °C. The wells were washed with 200 µl HH buffer and incubated with 100 µl (20 $mgml^{-1}$) bovine serum albumin (BSA) for 2 h at

4 °C. Then, the wells were washed twice with 200 µl of HH buffer to remove unbound BSA. The LAB suspension (about 10^6 CFU/ml) was suspended in 10 mmol/l phosphate-buffered saline (PBS) buffer in the wells and incubated at 37 °C for 1 hour. After, the wells were washed 5 times by using 200 µl sterile citrate buffer to remove unbound bacteria it was incubated with 0.5% (v/v) Triton X-100. Viable bacteria adhered to mucin on MRS agar was determined. The experiments were performed in triplicate. The adhesion percentage was calculated using the following equation:

The adhesion (%) = (Adhered strains/ strains add to the well) ×100

3.4 The application of LAB as starter culture in Pak-Sian fermentation

The selected LAB starter was cultured in MRS broth, overnight at 37 °C. The cultures about 10^6 CFU/ml after inoculation was centrifuged at 12,000 g, 5 mins. The pellets were washed twice in saline (0.85% w/v NaCl) and re-suspended in saline for inoculation in fermented Pak-Sian product modification of Leal-Sánchez *et al*. (2003).

The fermented Pak-Sian preparation was carried out according to modification of Steinkraus (2018); Pak-Sian was trimmed of defective leaves, washed and wilted in the sun for 1 hours for water evaporation until the vegetable is flaccid. The Pak-Sian were massaged with salt Pak-Sian: salt (10:1) and washed with water for 2 times. Then, Pak-Sian were packed in containers include 3% (w/v) of salts (500 ml), water from washing sticky rice (500 ml) and Pak-Sian (500 ml). This study divide into 4 treatments (Table 14);

*Table **14*** The treatment of fermented Pak-Sian

Treatments	Starter culture
1	Control (no add starter culture)
2	*Lb. fermentum* SK324 (10^6 CFU/ml)
3	*Lb. brevis* SK 335 (10^6 CFU/ml)
4	Mixed starter culture *Lb. fermentum* SK324 (10^6 CFU/ml): *Lb. brevis* SK335 (10^6 CFU/ml) (1:1)

The sampling of fermented Pak-Sian was done on the days 0, 1, 2 and 3. Fermented Pak-Sian were analyzed for the following:

1) The pH of the sample was measured by a pH meter.

2) The titratable acidity (% lactic acid) was measured by titration with a standard solution of 0.1 M NaOH using phenolphthalein as an indicator (Xiong *et al*., 2012). The sample in this experiment was analyzed in triplicates.

3) The LAB count: viable count of LAB was determined by spread plate method using MRS agar. The serial dilution was carried out using sterile normal saline. Each appropriate dilution was inoculated with 100 µl on MRS agar and incubated at 37 °C for 48 h. The LAB count is expressed in log CFU/g (Sánchez *et al*., 2001). The sample in this experiment was analyzed in triplicates.

4) The total viable count was determined by plate count agar after incubation at 37 °C for 48-72 h. (B. Tamang *et al*., 2008)

5) The fatty acid analysis by using gas chromatography. Cell suspension of fermented Pak-Sian was filtation by using 0.45 µm nylon membrane and determined the short chain fatty acid included acetic acid, propionic acid and butyric acid by using gas chromatography (GC Varian CP-3800 FID-Detector), column DB-FFAP (30m x 0.25mm x 0.25µm), Agilent technologies USA. The temperature of column was used an initial at 80 °C, hold for 5 min and then increase to 170 °C at the rate 10 °C/min and hold for 0 min, then increase to 250 °C at the rate 30 °C/min and hold for 5 min. The temperature of injector was 250 °C, split 30:1 and the temperature of detector used at 250 °C with flow rate at 1.00 ml/min.

6) The sensory evaluation of fermented Pak-Sian in term of colour, smell, sour, texture, odor and overall acceptance by using 9-point hedonic scale modification according to Di Cagno *et al*. (2013). All 30 untrained panelists were participated with male and female, age ranged 21-50 years old and they were not refused to consume the fermented Pak-Sian. Twenty gram of sample included marinated water and Pak-Sian were served to panelists. Before evaluate the next sample, the panelist were washed their mouth and consume bread

3.5 Statistical analysis

The Data were calculated as the mean and standard deviation (mean±SD) of triplicates. The data were performed by using analysis of variance (ANOVA) at $P<0.05$ and Duncan' Multiple Range Test (DMRT) were determined significant different between mean value. All statistical analyzes were determined using SPSS software version 12.

CHAPTER IV
RESULTS AND DISCUSSION

4.1 The sampling of fermented Pak-Sian samples

To obtain samples, eight fermented Pak-Sian were purchase from local markets in Kalasin, Sakhon Nakhon, Khon Kaen and Maha Sarakham provinces. The samples were analyzed chemical and microbiological properties include pH, % NaCl and lactic acid bacteria (LAB) count (Table 15). The chemical and microbiological properties of eight sample shown significantly differences ($P \leq 0.05$) due to difference in recipe of fermented Pak-Sian products (Table 16). The pH values of fermented Pak-Sian were in the range of 3.62-4.72%. Fermented Pak-Sian product from Khon Kaen (KK1) and Maha Sarakham (MK1) were found to have low pH about 3.62 and 3.73, respectively. LAB count were in the 8.52-9.69 log CFU/g, the difference in LAB count due to the different fermentation time of the fermented Pak-Sian. The high LAB count have been found in Khon Kaen (KK1) and Maha Sarakham (MK1) because of taking 2 days fermentation time, this fermentation time may promote LAB to grow rapidly and high populations develop. From this study, it was found that the production of fermented Pak-Sian different percentage of salt in the rang 1.33-2.45%. The use of salt in the fermentation of Pak-Sian contribute to salty taste and also help to improve the texture and limit unwanted bacteria. Additionally, it can be seen that Khon Kaen (KK1) and Maha Sarakham (MK1) had lower % NaCl than other fermented Pak-Sian by 1.33 and 1.74%, recpectively. The use of salt production may affect the growth of LAB which is related to the LAB count value of Khon Kaen (KK1) and Maha Sarakham (MK1), which were higher than that of other fermented Pak-Sian. However, the salt concentration may effect on LAB count. Y. S. Chen *et al*. (2006) found the high salt concentration in Suan-tsai prevent growth of most bacteria in the product. Consistency with Susilowati *et al*. (2008) determined LAB count of pickled ginger prepared using different salt concentration, found that high concentration effect on reduction of LAB count. Furthermore, Tabatabaei-Yazdi, Alizadeh-Behbahani, and Mortazavi (2013) revealed during fermentation the high salt concentration were LAB count higher than the low salt.

Table ***15*** Chemical and microbiological properties of fermented Pak-Sian samples (Mean±SD)

Markets	pH	NaCl (%)	LAB count (log CFU/g)
Kalasin (KS1)	4.41±0.03^{b}	2.26±0.23^{b}	8.87±0.02^{c}
Kalasin (KS2)	4.46±0.03^{b}	2.45±0.07^{a}	8.72±0.02^{d}
Sakon Nakhon (SK1)	4.28±0.03^{c}	2.14±0.01^{b}	8.89±0.03^{c}
Sakon Nakhon (SK2)	4.26±0.03^{c}	1.62±0.08^{c}	8.58±0.08^{e}
Khon Kaen (KK1)	3.62±0.03^{e}	1.74±0.02^{c}	9.51±0.03^{b}
Khon Kaen (KK2)	4.72±0.03^{a}	2.19±0.02^{b}	8.91±0.02^{c}
Maha Sarakham (MK1)	3.73±0.03^{d}	1.33±0.01^{d}	9.69±0.07^{a}
Maha Sarakham (MK2)	3.76±0.03^{d}	2.21±0.01^{b}	8.52±0.05^{e}

$^{a, b, c,d\cdots}$ superscripts of different letters in the column indicate significant difference ($P \leq 0.05$)

*Table **16*** Components for production of fermented Pak-Sian

Markets	Components	Fermentation time (Days)
Kalasin (KS1)	Salt, Sticky rice, Water from washing rice (paddy), Water	1
Kalasin (KS2)	Salt, Paddy, Paddy boiled rice water, water	1
Sakon Nakhon (SK1)	Salt, Sticky rice, Monosodium glutamate, Water	1
Sakon Nakhon (SK2)	Salt, Sticky rice, Water from washing rice, water	1
Khon Kaen (KK1)	Salt, Sugar, Water from washing rice (sticky), Water, Monosodium glutamate	2
Khon Kaen (KK2)	Salt, Water from washing rice (sticky), Water	1
Maha Sarakham (MK1)	Salt, Sticky rice, Water from washing rice (sticky), Water, Monosodium glutamate	2
Maha Sarakham (MK2)	Salt, Water from washing rice (sticky), Water, Monosodium glutamate	1

**The data is obtained from interviews

4.2 Identification of LAB isolates

The research aimed to isolate and identify LAB from fermented Pak-Sian samples and carry out studies on probiotic properties. Eight samples were collected from local markets in Kalasin, Khon Kaen, Sakon Nakhon and Maha Sarakham Provinces. LAB were purified by streaking on MRS agar, and bromocresol purple (0.05%) was used as pH indicator. The putative LAB were selected based on the yellow zone surrounded colony, catalase-negative and gram-positive characters. A

total of 234 presumptive LAB isolates were isolated from eight markets. The 234 isolates were screened for the presumptive LAB isolates by SDS-PAGE, using whole-cell protein patterns. The consideration of protein pattern base on the clearance, thickness position and number of bands (Jin *et al*., 2008). In this study, whole-cell protein patterns were selected by considering protein patterns that are expected the same patterns and different patterns. This study showed 61 isolates (patterns) from 234 isolates (Table 17 and Appendices E). Then, 61 isolates were identified using 16S rDNA sequencing. The sequence alignments of DNA sequences were performed using CLUSTAL OMEGA. The homology searches of DNA sequence were done by the BLAST alignment. However, the result showed the different patterns were identified as the same species based on 16s rDNA sequences. In detail, Kalasin markets (KS1 and KS2) were 5 and 8 the difference of whole cell protein, respectively, and their 16S rDNA sequences showed that the different patterns could be identified as the same species (Table 18). This study consistency with (Jin *et al*., 2008), investigated the diversity of LAB in Korean rice wine by using SDS-PAGE and identified based on 16S rRNA sequences, the different patterns showed the same species by 16S rRNA gene analysis. Sakon Nakhon markets (SK1; A, B, C, D patterns) were different the clearance, thickness position protein pattern that they were difference strain. However, the different protein patterns (SK1; A, E, F patterns) reveal the same species which provides the same results as Khon Kaen (KK1 and KK2) and Maha Sarakham (MK1 and MK2) markets. This study showed the presence of 17 bacterial isolates based on differences in 16S rDNA sequences.; *P. pentosaceus* KS12, *Pediococcus* sp. KS215, *P. pentosaceus* KS218, *P. pentosaceus* KS230, *Lb. plantarum* SK321, *Lb. fermentum* SK324, *P. pentosaceus* SK337, *Lb. brevis* SK335, *Lb. fermentum* SK48, *W. cibraria* SK415, *W. cibraria* SK432, *Lb. fermentum* SK434, *Lb. plantarum* KK53, *Lb. plantarum* KK518, *P. pentosaceus* MK74, *Lb. plantarum* MK711 and *Lb. plantarum* MK 724 (Table 19). A total of 17 strains that were selected are *P. pentosaceus Lb. plantarum, Lb. fermentum, Lb. brevis, Lb. fermentum* and *W. cibraria*, which was consistent with previous studies which found *Lb. brevis, P. pentosaceus* and *Lb. plantarum* in fermented Pak-Sian (Somboon Tanasupawat *et al*., 1992; S Tanasupawat & Komagata, 1995). Meanwhile, *W. cibraria* and *Lb. fermentum* were not reported in the product. The commonly present strains in the

fermented Pak-Sian were *P. pentosaceus* and *Lb. plantarum*. Our studies showed various LAB strains due to different samplings of product prepared by also different recipes. Each region of the Northeastern part of Thailand has a different recipe for the preparation of fermented Pak-Sian such as different salt concentration, fermentation time, fermented rice water and varieties of Pak-Sian, from different origins of Pak-Sian which may affect the diversity of LAB in fermented Pak Sian. Next, these 17 strains were evaluated for probiotic properties.

*Table **17*** Number of presumptive of LAB and the whole-cell protein patterns on SDS-PAGE

Markets	Number of isolates	Number of Whole cell protein patterns
Kalasin (KS1)	27	5
Kalasin (KS2)	27	8
Sakon Nakhon (SK1)	36	7
Sakon Nakhon (SK2)	12	7
Khon Kaen (KK1)	27	8
Khon Kaen (KK2)	27	14
Maha Sarakham (MK1)	36	5
Maha Sarakham (MK2)	44	7
Total	234	61

Table ***18*** Identification of LAB isolates selected on the basis of the whole cell protein patterns

Market	Whole-cell protein pattern	Bacterial species	Accession no.[a]	%identify[b]	Isolated no.
KS1	A	*P. pentosaceus* KCCM4073	CP020018.1	99	KS12
	B	*P. pentosaceus* KCCM4073	CP020018.1	100	KS18
	C	*P. pentosaceus* KCCM4073	CP020018.1	100	KS122
	D	*P. pentosaceus* KCCM4073	CP020018.1	100	KS126
	E	*P. pentosaceus* KCCM4073	CP020018.1	100	KS128
KS2	A	*P. pentosaceus* KCCM4073	CP020018.1	100	KS25
	B	*P. pentosaceus* KCCM4073	CP020018.1	99	KS28
	C	*P. pentosaceus* KCCM4073	CP020018.1	100	KS210
	D	*P. pentosaceus* ZZU223	AB831186.1	96	KS215
	E	*P. pentosaceus* KCCM4073	CP020018.1	99	KS218
	F	*P. pentosaceus* KCCM4073	CP020018.1	100	KS226
	G	*P. pentosaceus* KCCM4073	CP020018.1	100	KS230
	H	*P. pentosaceus* KCCM4073	CP020018.1	100	KS234

[a] Genbank accession no. on NCBI website (http://ncbi.nlm.mih.gov/pubmed)

[b] identity (%) from BLAST search

KS; Kalasin, SK; Sakon Nakhon, KK; Khon Kaen, MK; Maha Sarakham province

*Table **18*** Identification of LAB isolates selected on the basis of the whole cell protien patterns (continued)

Market	Whole-cell protein patterns	Blast result	Accession no.[a]	%identify[b]	Isolates no.
SK1	A	*P. pentosaceus* KCCM4073	CP020018.1	99	SK314
	B	*L. plantarum* KC28	CP026743.1	100	SK321
	C	*L. fermentum* LfQi6	CP025592.1	100	SK324
	D	*L. brevis* 1TP03-BL01	MG031209.1	99	SK335
	E	*P. pentosaceus* KCCM4073	CP020018.1	100	SK337
	F	*P. pentosaceus* KCCM4073	CP020018.1	100	SK340
	G	*L. plantarum* KC28	CP026743.1	100	SK344
SK2	A	*L. fermentum* BP-3.5	MF191690.1	99	SK48
	B	*L. fermentum* BP-3.5	MF191690.1	100	SK49
	C	*W. cibraria* BM2	CP027427.1	100	SK415
	D	*P. pentosaceus* KCCM4073	CP020018.1	99	SK420
	E	*P. pentosaceus* KCCM4073	CP020018.1	100	SK422
	F	*W. cibraria* BM2	CP027427.1	99	SK432
	G	*L. plantarum* KC28	CP026743.1	100	SK434

*Table **18*** Identification of LAB isolates selected on the basis of the whole cell protein patterns (continued)

Market	Whole-cell protein patterns	Blast result	Accession no.[a]	%identify[b]	Isolates no.
KK1	A	*L. plantarum* NRIC0413	AB362677.1	100	KK53
	B	*L. fermentum* LfQi6	CP025592.1	100	KK55
	C	*L. plantarum* NRIC0413	AB362677.1	99	KK513
	D	*P. pentosaceus* KCCM4073	CP020018.1	100	KK514
	E	*L. plantarum* KC28	CP026743.1	100	KK516
	F	*L. plantarum* KC28	CP026743.1	99	Kk518
	G	*L. plantarum* NRIC0413	AB362677.1	99	KK519
	H	*L. plantarum* KC28	CP026743.1	100	KK525
KK2	A	*L. fermentum* LfQi6	CP025592.1	100	KK69
	B	*L. fermentum* LfQi6	CP025592.1	99	KK626
	C	*L. plantarum* KC28	CP026743.1	100	KK665
	D	*L. plantarum* KC28	CP026743.1	99	KK651
	E	*L. plantarum* KC28	CP026743.1	100	KK612
	F	*L. plantarum* KC28	CP026743.1	100	KK67
	G	*L. plantarum* KC28	CP026743.1	100	KK611
	H	*P. pentosaceus* KCCM4073	CP020018.1	100	KK616
	I	*L. plantarum* KC28	CP026743.1	100	KK619

Table ***18*** Identification of LAB isolates selected on the basis of the whole cell protein patterns (continued)

Market	Whole-cell protein patterns	Blast result	Accession no.[a]	%identify[b]	Isolates no.
KK2	J	*P. pentosaceus* KCCM4073	CP020018.1	100	KK625
	K	*P. pentosaceus* KCCM4073	CP020018.1	100	KK624
	L	*L. plantarum* KC28	CP026743.1	100	KK625
	M	*L. plantarum* KC28	CP026743.1	100	KK627
	N	*L. plantarum* KC28	CP026743.1	100	KK643
MK1	A	*P. pentosaceus* JCM20459	LC311738.1	99	MK74
	B	*L. plantarum* KC28	CP026743.1	99	MK711
	C	*L. plantarum* KC28	CP026743.1	100	MK724
	D	*P. pentosaceus* KCCM4073	CP020018.1	99	MK725
	E	*P. pentosaceus* KCCM4073	CP020018.1	100	MK734
MK2	A	*L. fermentum* BP-3.5	MF191690.1	100	MK81
	B	*P. pentosaceus* KCCM4073	CP020018.1	100	MK86
	C	*P. pentosaceus* KCCM4073	CP020018.1	100	MK819
	D	*P. pentosaceus* KCCM4073	CP020018.1	100	MK832
	E	*P. pentosaceus* KCCM4073	CP020018.1	100	MK841
	F	*P. pentosaceus* KCCM4073	CP020018.1	100	MK844
	G	*P. pentosaceus* KCCM4073	CP020018.1	100	MK845

*Table **19*** Species of LAB isolated from fermented Pak-Sian samples obtained from 8 local markets

Isolated no.	Species	Accession no.	% identity
1	*P. pentosaceus* KS12	MH973191	99
2	*Pediococcus* sp. KS215	MH973183	96
3	*P. pentosaceus* KS218	MH973195	99
4	*P. pentosaceus* KS230	MH973184	100
5	*P. pentosaceus* SK337	MH973185	100
6	*P. pentosaceus* MK74	MH973182	99
7	*Lb. plantarum* SK321	MH973186	100
8	*Lb. plantarum* KK53	MH973187	100
9	*Lb. plantarum* KK518	MH973196	99
10	*Lb. plantarum* MK711	MH973192	99
11	*Lb. plantarum* MK724	MH973194	100
12	*Lb. brevis* SK335	MH973181	100
13	*Lb. fermentum* SK324	MH973188	100
14	*Lb. fermentum* SK48	MH973197	99
15	*Lb. fermentum* SK434	MH973189	100
16	*W. cibraria* SK 415	MH973193	100
17	*W. cibraria* SK432	MH973190	100

4.3 Study on probiotic properties

4.3.1 pH tolerance

The ability of 17 isolates to survive in MRS broth (pH 2.5) was studied. The results showed that 14 strains were able to survive at pH 2.5. The survival rate of the 14 isolates were in the range of 55.26-86.30%.The high survival rate (>80%) was shown by *Lb. plantarum* MK711 and *Lb. fermentum* SK434. Three strains that cannot survive at pH 2.5 were *Lb. fermentum* SK48, *W. cibraria SK415*, *W. cibraria* SK432. The results are summarized in Table 20. Therefore, only 14 isolates out of 17 were selected to study on bile salt tolerance and survival in simulated gastric and intestinal tract. Seventeen strains of LAB were isolated from fermented Pak-Sian and studied

for probiotic properties and evaluated for its potential to be used as starter culture. In this study pH 2.5 was used to screen strains for its ability to survive in acidic condition as LAB must be able to survive the acidic condition of human gastric juice (Pennacchia *et al*., 2004). The pH of stomach varies from 2.5-3.5 and can inhibit the microbes (W. H. Holzapfel *et al*., 1998), therefore stated authors investigated the use of PBS at the desired pH to screen strains for their ability to maintain viability in vivo when exposed to gastric juice (Argyri *et al*., 2013). Similary, stated authors Ramos, Thorsen, Schwan, and Jespersen (2013) used pH tolerance property as a criterion for selection of isolates for further experimentation. Our study showed only 14 strains (*P. pentosaceus* KS12 KS218 KS230 SK337 MK74, *Pedicoccus* sp. KS 215, *Lb. plantarum* SK321 KK53 KK518 MK711 MK724, *Lb. fermentum* SK324 SK434 and *Lb. brevis* SK335) were able to survive in pH 2.5. Similarly, previous studies Ryu and Chang (2013); Bao *et al*. (2010); Vera-Pingitore *et al*. (2016) reported that *Lb. plantarum* and *P. pentosaceus* isolated from kimchi were tolerance to pH 2.5 which may be due to exopolysacchaide production and thus increased bacterial cell viability. Exopoysaccharide has the ability to reduce the effect of low pH and decrease cell viability of many strains (Sabir, Beyatli, Cokmus, & Onal-Darilmaz, 2010; Yuksekdag & Aslim, 2010). Interestingly, most studies reported that *Lb. plantarum* isolated from fermented vegetable product were highly tolerant to pH 2.5 (Argyri *et al*., 2013; E. A. Choi & Chang, 2015; Vera-Pingitore *et al*., 2016). Then, 14 strains were further evaluate for probiotic properties. Our study showed only 14 strains were able to survive in 2.5 pH and they were further evaluated for probiotic properties.

*Table **20*** Survival rate (% sv) of 17 LAB isolates in pH 2.5 (Mean±SD)

LAB strains	Survival rate (%)
P. pentosaceus KS12	71.37±0.06^{e}
Pediocuccus sp. KS215	71.97±0.81^{e}
P. pentosaceus KS218	60.63±0.38^{g}
P. pentosaceus KS230	63.28±0.64^{f}
P. pentosaceus SK337	55.26±0.40^{i}
P. pentosaceus MK74	60.43±0.62^{g}
Lb. plantarum SK321	79.87±0.20^{c}
Lb. plantarum KK53	77.13±0.65^{d}
Lb. plantarum KK518	77.72±0.36^{d}
Lb. plantarum MK711	86.30±0.16^{a}
Lb. plantarum MK724	81.17±0.03^{b}
Lb. brevis SK335	60.34±0.80^{g}
Lb. fermentum SK324	56.62±0.58^{h}
Lb. fermentum SK48	00.00±0.00^{j}
Lb. fermentum SK434	80.07±0.09^{c}
W. cibraria SK 415	00.00±0.00^{j}
W. cibraria SK432	00.00±0.00^{j}

$^{a, b, c,d\cdots}$ superscripts of different letters in the column indicate significant difference ($P \leq 0.05$)

4.3.2 Bile salt tolerance test

The ability of 14 isolates to survive in bile salt was estimated using MRS broth including 0.3% oxall and incubated for 3 h, the results showed that 14 strains were able to survive the bile salt condition. The survival rates of the14 strains were in the range from 65.59 to 95.13%. *Lb. plantarum* KK518 were high survived rate while *Lb. fermentum* SK434 had the least survival rate. The results are summarized in Table 21. The significant property of lactic acid bacteria which validates its probiotic capability is its ability to resist the effects of bile salt (Y.-K. Lee & Salminen, 1995; Swain *et al.*, 2014). Bile acid is synthesized from cholesterol in the liver and is stored

in the gall bladder. The bile acid can degrade lipid and absorb vitamins insoluble in water. The volume of bile acid synthesized in humans is about 500-700 ml/day at a concentration of 0.3% (Morelli, 2000). Therefore, 0.3% bile salt was used in this study. Most of the research used 0.3% (w/v) bile salt which mimics human bile salt (P. Chen *et al*., 2014; K. W. Lee *et al*., 2016; W.-H. Lin, Hwang, Chen, & Tsen, 2006; Taranto *et al*., 2006) stated authors Taranto *et al*. (2006) reported the effect of bile acid on the cell membrane of microbes and found that bile salt destroyed the lipid bilayer structure of the cell membrane and inhibited sugar transport into cell which causes cell death. This study found that some *Lactobacillus* strains were tolerant to bile salt (survival rate over 80%). Similarly, Argyri *et al*. (2013) reported that some *Lactobacillus* strains were tolerant to bile salt which was consistent with the bile salt hydrolase activity of some strains. Some strains showed partial bile salt hydrolysis. Bile salt hydrolase can hydrolyze bile salt and lead to reduced toxicity (Noriega, Gueimonde, Sánchez, Margolles, & de los Reyes-Gavilán, 2004) stated authors Vera-Pingitore *et al*. (2016) investigated the bile salt properties of lactic acid bacteria isolated from a fermented plant which showed good cell viability. The ability to tolerate bile salt is an important characteristic of probiotic bacteria, as it will help the bacteria to survive in the digestive system and aid adsorption into the gastrointestinal tract. Our studies found 14 strains that can survive in bile salt condition.

*Table **21*** Survival rate (% sv) of 14 LAB isolates in bile salt (Mean±SD)

LAB strains	Survival rate (%)
P. pentosaceus KS12	74.04±0.71[f]
Pediococcus sp. KS215	76.60±0.86[e]
P. pentosaceus KS218	73.48±0.69[f]
P. pentosaceus KS230	73.47±0.97[f]
P. pentosaceus SK337	76.22±0.35[e]
P. pentosaceus MK74	79.26±0.16[d]
Lb. plantarum SK321	71.37±1.01[g]
Lb. plantarum KK53	95.13±0.89[a]
Lb. plantarum KK518	89.17±0.62[b]
Lb. plantarum MK711	87.97±0.86[c]
Lb. plantarum MK724	89.74±1.04[b]
Lb. brevis SK335	88.70±0.14b[c]
Lb. fermentum SK324	87.91±0.81[c]
Lb. fermentum SK434	65.59±0.18[h]

[a, b, c,d…] superscripts of different latters in the column indicate significant difference ($P \leq 0.05$)

4.3.3 Survival in simulated gastric and intestinal tract

In this study, the simulated gastric was studied using MRS broth added 0.3% pepsin and 0.5% (w/v) NaCl, pH 3.0 was used. The results showed that the survival rates of 14 isolates were in the range of 16.56-81.45%. The high survival rate (>80%) as shown by *Lb. plantarum* KK53, and *Lb. fermentum* SK434. The results are summarized in Table 22. The properties of probiotic bacteria that should be considered are tolerance to gastric acid because probiotic bacteria are taken along with fermented food. After the food is consumed, the probiotic bacteria pass through the stomach and encounters the gastric juice secreted into the stomach that cause reduction of the pH in the stomach. The digestion of food in the human stomach takes approximately 3 h, and the pH of the stomach varies from 2.5 to 3.5 and inturn inhibits the microbes (W. H. Holzapfel *et al*., 1998). Therefore, the probiotic bacteria

must be resistant to the gastric acid in the stomach, after which they move through into intestine tract (Henriksson *et al*., 1999). In the study gastric acid (pepsin) at pH 2.5 was used. The results showed that the survival rate of the 14 isolates were in the range of 20.60-85.72%. Lourens-Hattingh and Viljoen (2001) showed that probiotic microbes need to be viable and tolerant to the stressful condition of the stomach and the anti-microbial activity of bacteria in gut. Notable resistance in simulated gastric juice was shown by *P. pentasaceus* KS12, *Lb. fermentum* SK434, *Lb. plantarum* KK53 and *Lb. plantarum* MK711. The results are summarized in Table 22. The results of the present are in accordance with (Zhang *et al*., 2016), *Lactobacilli* strain can survive in simulated gastrointestinal environment, with a survival rate of over 90%. Tulini *et al*. (2013) reported that *Lactobacillus paraplatarum* FT 259 can tolerate simulated gastric juice and remain viable. Similarly, H. Lee *et al*. (2011) reported *Lactobacillus* strain can survive in the presence of pepsin at pH 2.5.

The intestinal tolerance was assessed using the simulated intestinal (0.1% w/v pancreatin in sterile 0.5% w/v NaCl, pH 8). All 14 strains were able to survive in the simulated intestinal conditions. The results showed that the survival rates of 14 isolates were in the range of 86.16-98.23%. *P. pentosaceus* MK74 showed the highest survival rate while *Lb. plantarum* KK518 had the least survival rate when cultivated in the simulated intestinal condition. (Table 22). From the experiment, it can be seen that the survival rate of 14 strains was quite high value may due to this study does not investigate imitation of digestion because not bringing the bacteria survive from simulated gastric juice to continue study in the intestinal tract. The probiotic properties should consider the survival in intestinal tract when bacteria survival from stomach condition and then passes through the intestinal tract. The pH of the intestinal tract is about pH 7.0-8.0 (De Beer, Johnston, & Wilson, 1935) and pancreatic juice is secreted into the small intestine each day having a salt content not less than 0.5% (Charteris *et al*., 1998). Lapsiri *et al*. (2011) studied on the tolerance small intestinal juice with the bile salt (pH 8) of *Lactobacillus plantarum* and viable cell counts after 4 hours, all strains (TISTR 2073, TISTR 2077 and TISTR 2081) were tolerance in simulated intestinal juice and the survival rate were 84.90, 89.96 and 89.31%, respectively. Charteris *et al*. (1998) studied on small intestinal tolerance of probiotic

Lactobacillus and *Bifidobacterium* species by using pancreatic juice (pH 8) and NaCl (0.5%), *Lactobacillus* and *Bifidobacterium* species can survive in the simulated small intestinal juice.

*Table **22*** Survival rate (% sv) of 14 LAB isolates in simulated gastric and intestinal tract (Mean±SD)

LAB strains	simulated gastric Survival rate (%)	intestinal tract Survival rate (%)
P. pentosaceus KS12	67.21±0.35^{c}	90.59±0.81^{d}
Pediococcus sp. KS215	46.55±0.42^{e}	96.45±1.06^{abc}
P. pentosaceus KS218	16.56±0.56^{j}	97.25±0.74^{abc}
P. pentosaceus KS230	30.11±0.19^{g}	96.04±0.34^{bc}
P. pentosaceus SK337	20.33±0.81^{i}	97.30±1.03^{abc}
P. pentosaceus MK74	31.47±0.31^{g}	98.23±1.17^{a}
Lb. plantarum SK321	36.66±0.38^{f}	96.88±0.93^{abc}
Lb. plantarum KK53	81.45±0.36^{a}	95.78±0.66^{c}
Lb. plantarum KK518	21.02±0.63^{i}	86.16±0.83^{e}
Lb. plantarum MK711	77.43±0.60^{b}	96.76±0.14^{abc}
Lb. plantarum MK724	26.64±0.76^{h}	97.85±0.71^{ab}
Lb. brevis SK335	57.29±0.53^{d}	97.83±0.55^{ab}
Lb. fermentum SK324	46.59±0.63^{e}	97.55±0.27^{abc}
Lb. fermentum SK434	80.12±0.93^{a}	97.08±0.26^{abc}

$^{a, b, c,d\ldots}$ superscripts of different letters in the column indicate significant difference ($P \leq 0.05$)

4.3.4 Antibiotic susceptibility

The antibiotic susceptibility of 14 LAB isolates was determined using 6 antibiotic discs: ampicillin (10 µg), azithromycin (15 µg), rifampicin (5 µg), chloramphenicol (30 µg), streptomycin (25 µg) and vancomycin (30 µg). The results showed that all of the 14 species of LAB were resistant to antibiotics except some species such as *Lb. plantarum* SK321 KK518, *Lb. fermentum* SK324. *Lb. brevis*

SK*335* that showed moderate susceptibility to cholamphenical and *Lb. fermentum* SK434, *Lb. plantarum* KK53, KK518, MK724 were moderately susceptible to rifampicin. Meanwhile, *Lb. plantarum* SK321, *Lb. fermentum* SK 434 and *Lb. plantarum* MK711, MK724 were susceptibility to ampicillin (Table 23). Some LAB strains showed resistance to all 6 antibiotics whereas, *Lactobacillus plantarum* was susceptible to ampicillin which was in agreement with the results of K. W. Lee *et al*. (2016) who studied the antibiotic susceptibility of *Lb. plantarum* isolated from Kimchi and found that it was susceptible to ampicillin. In this study all of 14 isolates were resistant to streptomycin which was consistent with the findings of stated authors Zhang *et al*. (2016) who showed that the *Lactobacillus* strains from milk cheese were resistant to streptomycin. The observed 14 species showed resistance to vancomycin which was in agreement with the findings of Karasu *et al*. (2010) who found that the *Lb. plantarum* strain isolated from traditionally produced fermented vegetable showed resistance against vancomycin which consistent with E. A. Choi and Chang (2015) found *Lb. plantarum* was resistant to vancomycin. Tulumoğlu, Kaya, and Şimşek (2014) showed *Lb. fermentum* was resistant to vancomysin. This results revealed that *Lactobacillus* strain have resistance to vancomycin which support the finding of Tulini *et al*. (2013) and Salminen *et al*. (1998) who found vancomycin resistance is an instrinsic feature of lactobacilli. The genus *Pediococcus a*nd *Lactobacillus* are intrinsically resistant to the glycopeptide such as vancomycin which is a common feature among lactic acid bacteria (Hummel, Hertel, Holzapfel, & Franz, 2007). The safety of probiotic bacteria should be considered with regards to antibiotic sensitivity because antibiotics are used for the prevention and control of intestinal infection (El-Naggar, 2004) and the probiotic bacteria with antibiotic resistant gene might survive. The disadvantage of antibiotic resistance is the transfer of antibiotic resistance gene on plasmid to other bacteria via conjugation (Cebeci & Gürakan, 2003). However, this study did not investigate whether antibiotic resistance genes are present on chromosomes or on plasmids.

4.3.5 Antimicrobial activity of LAB

The antimicrobial activity of 14 LAB species was determined using the agar spot test against 4 pathogenic bacterial strains; *S. aureus*, *S. typhymurium*, *E. coli*

and *B. cereus*. The results showed that 14 species inhibited all tested pathogens. Most of the species showed effective inhibition against *E. coli*. The data is summarized in Table 24. The antimicrobial activity of 14 LAB species exhibited widest clear zone of *S. typhymurium* and *E. coli* (gram-negative) more than *S. aureus* and *B. cereus* (gram-positive) which gram-positive have thickness cell wall include peptidoglycan about 50-60% of cell wall while Gram-negative have a thin layer cell wall. According to Hwanhlem *et al*. (2011) found the antoganistics of LAB toward *E.coli* and *Samonella* sp. more than *S. aureus* which the antoganistics activity due to organic acid. J. P. Tamang et al. (2009) reported *Lb. plantarum*, *Lb. brevis* and *P. pentosaceus* isolated from fermented bamboo inhibited a pathogenic gram-negative and gram-positive microorganism, found *Lb. plantarum* IB948 produce a bacteriocin inhibit *S. aureus*. Ramos *et al*. (2013) revealed *Lb. fermentum*, *Lb. plantarum* and *Lb. brevis* isolated from Bazilian fermented product able to againt pathogenic bacteria; *S. aureus* and *L. monocytogenes*. H. Lee *et al*. (2011) the antimicrobial properties against pathogenic bacteria of *Lactobacillus* strains are organic acid, H_2O_2 and bacteriocin. Moreover, Saelim, Jampaphaeng, and Maneerat (2017) mentioned, mechanism of organic acid on inhibiting of microorganism can occur due to undissociated form organic acid, which it can diffuse through the cell membrane and into the cytoplasm and dissociate into anions and protons, releasing proton ions effect on pH of internal cell to decrease and induce to destroy of proton motive force and inhibiting substrate transport mechanism. E. A. Choi and Chang (2015) mentioned the beneficial requirements of probiotics starter should be broad antimicrobial spectrum against gram-negative and gram-positive pathogenic bacteria. The antimicrobial activities are important criteria for the selection of probiotic strains which indicate antagonism between colonic flora and pathogenic bacteria. The antibacterial substances can inhibit pathogenic bacteria such as bacteriocin, low molecular weight metabolites; organic acid, fatty acids, hydrogen peroxide, and diacetyl (Caplice & Fitzgerald, 1999). Furthermore, lactic acid from LAB isolates during fermentation can combine with bile salts and lead to the inhibition of the growth of gram-negative pathogenic bacteria (Begley, Hill, & Gahan, 2006). Antagonistic activity is important to prevent the infection of undesirable bacteria, where the antagonist activity of the 14 species of LAB was studied against the representatives of food pathogens. LAB can inhibit pathogenic

bacteria in fermented vegetable product which is an important role in the preservation of fermented vegetable product and provides safety for consumption by the consumer (J. P. Tamang *et al*., 2009).

*Table **23*** The antibiotic susceptibility of 14 LAB species

Species	Amp (10 µg)	Azm (15 µg)	RD (5 µg)	C (30 µg)	S (10 µg)	V (30 µg)
P. pentosaceus KS12	R	R	R	R	R	R
Pediococcus sp. KS215	R	R	R	R	R	R
P. pentosaceus KS218	R	R	R	R	R	R
P. pentosaceus KS230	R	R	R	R	R	R
Lb. plantarum SK321	S	R	R	M	R	R
Lb. fermentum SK324	M	R	R	M	R	R
P. pentosaceus SK337	R	R	R	R	R	R
Lb. brevis SK335	R	R	R	M	R	R
Lb. fermentum SK434	S	R	M	S	R	R
Lb. plantarum KK53	R	R	M	S	R	R
*Lb. plantarum*KK518	M	R	M	M	R	R
P. pentosaceus MK74	R	R	R	R	R	R
Lb. plantarum MK711	S	R	R	S	R	R
Lb. plantarum MK724	S	M	M	S	R	R

Amp, Ampicilin; Azm, Azithromycin; RD, Rifampicin; C, Chloramphenical; S,Streptomycin; V, Vancomycin. Inhibition zones are reported in term of resistant (R), moderate susceptibility (M) and susceptible (S)

*Table **24*** Antimicrobial activity of 14 LAB species (Mean±SD)

Species	Diameter of clear zone (mm)			
	S. aureus	*S. typhymurium*	*E. coli*	*B. cereus*
P. pentosaceus KS12	7.5 ±0.75 +	14.2±0.34 ++	21.3±0.2+++	6.76 ±0.11 +
Pediococcus sp. KS215	6.67 ±0.25 +	19.15 ±0.23++	26.33 ±0.23 +++	8.76 ±0.24 +
P. pentosaceus KS218	10.28 ±0.91 ++	17.93 ±0.28++	19.08 ±0.81 ++	6.6 ±0.39 +
P. pentosaceus KS230	6.57 ±0.28 +	16.1 ±0.71 ++	19.16 ±0.70 ++	6.83 ±0.59 +
Lb.plantarum SK321	10.85 ±0.47 ++	19.55 ±0.22 ++	20.78 ±0.97 +++	10.31 ±0.18 ++
Lb. fermentum SK324	5.08 ±0.75 +	19.53 ±0.52++	23.13 ±0.60 +++	6.78 ±0.30 +
P.pentosaceus SK337	9.13 ±0.40 +	16.51 ±0.07++	19.56 ±0.64 ++	9.15 ±0.78 +
Lb. brevis SK335	8.11 ±0.28 +	19.35 ±0.90 ++	20.63 ±0.64 +++	10.58 ±0.79 ++
Lb. fermentum SK434	12.33 ±0.57 ++	19.23±0.75 ++	21.56±0.48 +++	13.46 ±0.98 ++
*Lb. plantarumKK*53	4.26 ±0.66 +	16.35 ±0.67 ++	26.76 ±0.47 +++	4.78 ±0.78 +
*Lb. plantarum*KK518	11.23 ±0.06 ++	16.70 ±0.58 ++	20.38±0.75 +++	18.88±0.33 ++
P. pentosaceus MK74	9.9 ±0.25 +	16.47 ±0.95 ++	21.43 ±0.32 +++	8.55 ±0.13 +
Lb. plantarum MK711	11.8 ±0.91 ++	19.28 ±0.53 ++	18.56 ±0.50 ++	10.98±0.51 ++
Lb. plantarum MK724	12.6±0.56++	22.13 ±0.85 +++	19.48 ±0.40 ++	9.73 ±0.84 +

+ shown as diameter of inhibition zone less than 10 mm; ++ shown as diameter of inhibition zone, 10 to 20; +++ shown as diameter of inhibition zone, above 20 mm

4.3.6 Detection of biogenic amine production

Biogenic amine production of 14 species was determined using decarboxylase medium containing 1% each of amino acid (histidine, lysine, ornithine and tyrosine). The result showed that the LAB strains did not produce biogenic amine (Table 25) . The selection of probiotic LAB for utilization in food product should consider on the safety. The determination of biogenic amine and haemolytic activity were considered a safety aspect for the selection of probiotic strains. Biogenic amines are non-volatile low molecular weight nitrogenous organic bases, derived through decarboxylation of amino acids. Some species of LAB can produce biogenic amines. The foods containing biogenic amines are responsible for food poisoning (Buckenhüskes, 1993; Spano *et al*., 2010). The biogenic amines are commonly found in lactic acid bacteria fermented vegetables and it is desirable that their presence in the food product is minimized or nil therefore by carrying out investigation on biogenic amines, we ensure the food safety of our product. The genera of *Enterobacteriaceae* and *Bacillaceae* as well as species of *Lactobacillus*, *Pediococcus* and *Streptococcus* are reported to exhibit decarboxylation of one or more amino acids. The major amines found in higher concentration in foods are histamine, tyramine, putrescine and cadaverine. The biogenic amines found in fermented vegetables are as ethanolamine, putrescine, cadaverine, permidine, pheylethylamine, tyramine and histamine (Buckenhüskes, 1993). Our results show that 14 LAB species did not synthesize biogenic compound. Biogenic amine are found in a wide ranges of food, especially in protein-rich and fermented food (Shalaby, 1996). In this study not found biogenic amine production due to major ingredients of fermented Pak-Sian do not contain high level of biogenic amine precursor. This result was in correlation to the result of Ji *et al*. (2013) where, five strains of *Lactobacillus plantarum* isolated from Korean kimchi did not produce biogenic compound which indicates the safety of the product. Whereas, H. Lee *et al*. (2011) showed *Lb. plantarum* and *Lb. saki* isolated from Kimchi were positive with tyrosine which these strains not safe and should not be used. Ji *et al*. (2013) explained biogenic amine properties of the same species may provide different result which this properties may related to strain specific. J. Yang *et al*. (2014) studies the safety properties of probiotic such as biogenic amine production, that found *W. viridescens, W. confuse, W. cibraria* and *Lb. plantarum* not

produce biogenic amine, indicate that these LAB strains are safe to use in fermented product. In accordance with J. P. Tamang *et al*. (2009) mentioned LAB isolated from Himalayan fermented vegetable not found biogenic amine production which the good properties for development as starter culture.

4.3.7 Haemolytic activity

Haemolytic activity of 14 LAB species was determined using 5% sheep blood. Only 8 LAB species showed γ-haemolysis (no haemolysis) such as *P. pentosaceus* (KS12, KS230, SK337, MK74), *Pediococcus* sp. KS215, *Lb. fermentum* (SK324, SK434) and *Lb. brevis* SK335. All strains of *Lb. plantarum* showed α-haemolysis (Table 25) . This result not found β-haemolysis. This study analogous report of Hawaz (2014), LAB strains showed the most of LAB strains were γ, α-haemolysis and did not found β-haemolysis. Next, only these 8 strains were evaluated for the next experiment. Only 8 LAB species out of 14 showed γ-haemolysis (no haemolysis) which is a good indication of their acceptability and their potential for the possible development as probiotic starter culture (Hawaz, 2014; J. P. Tamang *et al*., 2009). LAB are considered as Generally Recognized as Safe (GRAS) microorganism (Klaenhammer *et al*., 2005; Silva *et al*., 2002). However, the safety of LAB should be evaluated before consideration for use as a probiotic starter culture. Several studies were evaluated the safety property of LAB isolated from fermented product, found *Lb. plantarum*, *Lb. fermentum Lb. pentosus* and *Lb. paracasa*i not found haemolytic activity, this test indicate the safety and considered as probiotic candidates (Argyri *et al*., 2013; Ji *et al*., 2013; Rushdy & Gomaa, 2013). Our results showed that only 8 LAB species which showed no haemolysis activity were further studied for the adhesion capacity to pig intestinal mucin.

*Table **25*** The Biogenic amine and haemolytic activity of 14 species LAB

Species	Biogenic amine	Haemolysis activity
P. pentosaceus KS12	-	γ
Pediococcus sp. KS215	-	γ
P. pentosaceus KS218	-	α
P. pentosaceus KS230	-	γ
Lb. plantarum SK321	-	α
Lb. fermentum SK324	-	γ
P. pentosaceus SK337	-	γ
Lb. brevis SK335	-	γ
Lb. fermentum SK434	-	γ
*Lb. plantarum KK*53	-	α
Lb. plantarum KK518	-	α
P. pentosaceus MK74	-	γ
Lb. plantarum MK711	-	α
Lb. plantarum MK724	-	α

4.3.8 Adhesion capacity to pig intestinal mucin

The adhesion capacity to pig intestinal mucin was evaluated for the 8 selected LAB species which did not exhibit any haemolytic activity. All 6 LAB species showed very low percentage of adhesion capacity (0-0.25%) such as *P. pentosaceus* KS 12, KS 215, KS230, SK337, MK74, and *Lb. fermentum* SK 434 (Figure 5). However, *Lb. fermentum* SK324 and *Lb. brevis* SK 335 exhibited high percentage of adhesion capacity (2.39% and 2.34%, respectively). This finding revealed that *Lb. fermentum* SK324 and *Lb. brevis* SK335 possessed good mucin adhesion properties. Meanwhile, other LAB strains showed slight adhesion properties. *P. pentosaceus* KS12 and SK337 were not able to adhere to mucins. Previous studies investigated the adhesion capacity by using pig intestinal mucin (Carasi *et al*., 2014; Li *et al*., 2008). Valeriano *et al*. (2014) investigated mucin adhesion of *Lactobacillus mucosae* species, *Lactobacillus mucosae* species have shown good mucin adhesion

when compared with a commercial strain (LGG). Martín *et al*. (2009) isolated lactobacilli from sow milk, the results showed *Lb. reuteri*, *Lb. plantarum*, *Lb. salivarius*, *W. paramesenteroides*, *Lb paraplantarum*, *Lb. brevis* and then they were investigated adherence to porcine mucin, all strains showed adhesion value about 0.03-12.39 % while *Lb. brevis* displayed the lowest adhesion value (0.03%). Olivares, Díaz-Ropero, Martín, Rodríguez, and Xaus (2006) studied on adhesion properties of *Lactobacillus* strains isolated from breast milk by using porcine mucin, *Lactobacillus* strains (*L. gasseri* CECT5714, *Lb. gasseri* CECT5715, *Lb. fermentum*, *Lb. gasseri* CECT5716, *L*b. *salivarius* CECT5713, *Lb. coryniformis*, *Lb. gasseri* CECT5711) were able to adhere to porcine mucin (about 0.6-1.6%) while *Lb. brevis* is about 1.1 %. In addition, there is also a study on the using of caco-2 cell in adhesion studies. This study corresponds to Tulumoğlu *et al*. (2014) studied adhesion properties of *Lb. fermentum* strains by using caco-2 cell, found that adhered 2-14% to caco-2 cell which different strains of *Lb. fermentum* revealed different adhesion value. Ramos *et al*. (2013) reported adhesion properties *Lb. fermentum* FFC199 and *Lb. brevis* CH 58 isolates showed ability to adhered caco-2 cell, the adhesion value 0.9 and 0.8%, respectively. Adherence properties are the one of the most important criteria for selection of probiotic bacteria. Adhesion of probiotic to the intestinal surface and the colonization of human GI-tract is an important requirement for probiotic activity. The mechanism of adherence of probiotics and pathogen microorganism onto the intestinal surface is the same therefore competition is observed, this in turn causes the pathogenic microorganism's inability to adhere and is therefore disposed from the GI-tract (Saarela *et al*., 2000). Moreover, the adhesion requires an interaction with the mucus surface leading to the contact with the gut related lymphoid tissue mediating local and systemic immune effect.

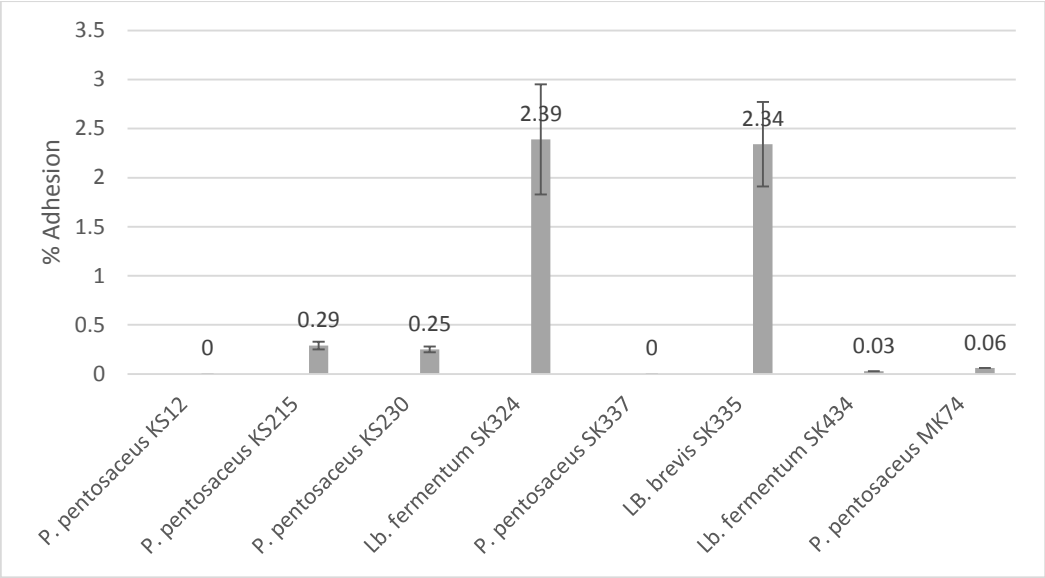


Figure **5** Percent adhesion of 8 strains LAB

4.4 The application of LAB as starter culture in Pak-Sian fermentation

Probiotic microorganisms has been accepted as safe microorganisms (GRAS; Generally Recognized As Safe). The criteria for the selection of probiotic bacteria pertaining to safety, functionality and technological features are: acid and bile stability, adherence to human intestinal cell, persistence in the human intestinal tract, production of antimicrobial substance and antagonism against pathogenic bacteria (Mattila-Sandholm *et al*., 2002; Saarela *et al*., 2000). Likewise, J. Yang *et al*. (2014) and Ji *et al*. (2013) report that a suitable starter culture should be selected based on safety properties such as no biogenic amine production and no haemolysis activity. This study investigated fermentation of fermented Pak-Sian by using probiotic starter culture. All 8 strains showed ability to survive in bile salt and pH tolerance, ability to survive in gastric and intestinal tract, antagonistic activity against pathogenic bacteria, no haemolytic activity and biogenic amine production. The mucin adhesion capacity of two strains (*Lb*. *fermentum* SK324, *Lb*. *brevis* SK335 was higher than other strains. Thus, this study selected two strain (*Lb*. *fermentum* SK324, *Lb*. *brevis* SK335) to be used as starter culture fermentation of Pak-Sian was divided into 4 treatments; inoculation with *Lb*. *fermentum* SK324, inoculation with *Lb*. *brevis* SK335, mixed starter culture (*Lb*. *fermentum* SK324: *Lb*. *brevis* SK335; 1:1) and control (no addition of starter culture). Pak-Sian was fermented for 3 days and every day (0, 1, 2 and 3

days) , the results of the following was checked; pH, lactic acid, LAB count, TPC count, and short chain fatty acid.

4.4.1 pH value

The study on pH value of fermented Pak-Sian showed initial pH value of 6.01-6.09 which was not significant (P>0.05) between treatments. The final pH of fermented Pak-Sian ranged from 4.18 to 4.32. During fermentation it was found that the pH tended to decrease continuously (Figure 5). On the 1st day of fermentation, the reduction in pH was significantly more rapid in the treatment which utilized starter culture when compared to control (no addition of starter culture) while control treatment showed a slow reduction which was in agreement with Tolonen *et al*. (2002) and Xiong, Li, Guan, Peng, and Xie (2014). The decreased acidity due to the growth of LAB which have ability to convert sugar into lactic acid has an effect on the decreasing of pH value. This study showed that the fermentation of Pak-Sian using *Lb. fermentum* SK324 had a low pH of about 4.18 in the end product. Oguntoyinbo *et al*. (2016) reported that the starter culture has the ability to rapidly produce acid which results in the decrease of pH in food. A pH value of less than 4.2 is the main indicator of safety with regards to fermented foods. This justification is in agreement with Yan *et al*. (2008) who revealed that the increase in lactic acid and decrease in pH inhibited the *Enterobacteria* count in cabbage. Beganović *et al*. (2011) study on sauerkraut production using *Lactobacillus plantarum* L4 and *Leuconostoc mesenteroides* LMG 7954 as starter culture indicated that the starter culture was responsible for rapidly decreasing pH and can inhibit the food-spoiling bacteria. Gardner, Savard, Obermeier, Caldwell, and Champagne (2001) reported that sauerkraut fermentation contains 2 type of LAB involved in fermentation: heterofermentative and homofermentative. During the first stage of fermentation, hetrofermentative bacteria grows and produces lactic acid, acetic acid, ethanoland CO_2 resulting in the rapid decrease of pH. Then, homofermentative LAB continues the fermentation to decrease pH. In correlation with the aforementioned results, as this study uses *Lb. fermentum* SK324 and *Lb. brevis* SK335 which are heterofermentative LAB may help reduce the pH of fermented Pak-Sian in first stage more rapidly than control treatment. This study showed that the pH value of starter culture treatment

was lower than the control treatment which is in agreement with J. Yang *et al*. (2014) who investigated appropriate starter cultures for fermentation of Korean leek product. They found that the pH value of spontaneous fermentation decreased after 48 h. to pH 4.40 and was lower than control treatment. Moreover, the decrease in the acidity of fermented Pak-Sian is associated with an increase in lactic acid bacteria which can convert sugar to lactic acid (Klaenhammer & Kullen, 1999)

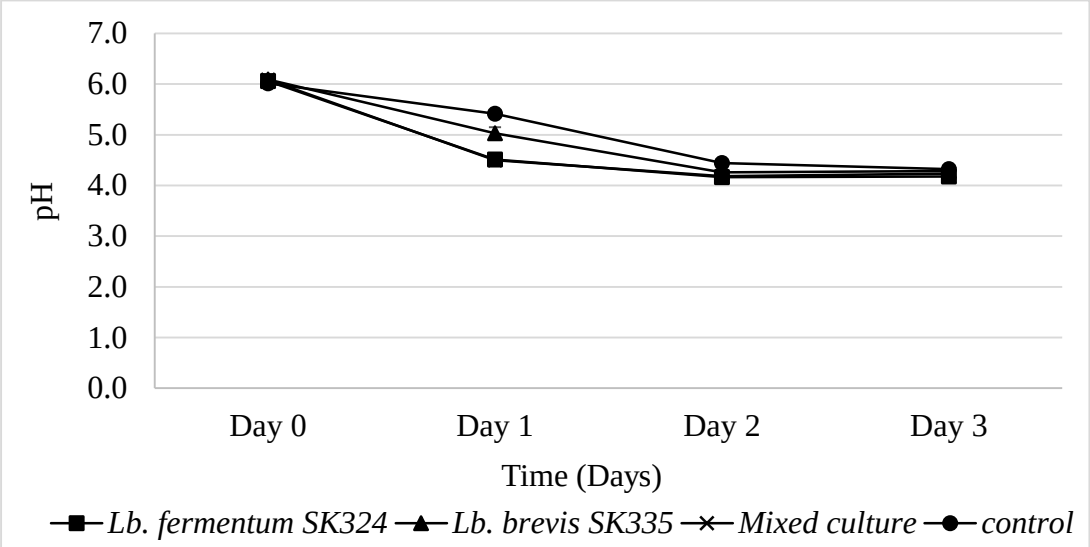


*Figure **6** pH value of fermented Pak-Sian*

4.4.2 Lactic acid bacteria (LAB) count

The LAB count of fermented Pak-Sian at 0, 1, 2 and 3 days are shown in Figure 7. This study showed that in the control treatment the LAB count was lower than the starter culture treatment ($P \leq 0.05$) and LAB count tended to increase. While on the 3rd day of fermentation, all treatments were not significant in LAB count ($P > 0.05$) . LAB count were ranged between 8.13-8.18 log CFU/g. In the final fermentation process, the value of LAB count was over 10^6 CFU/g. Beganović *et al*. (2011) and (Shah, 2000) reported that the probiotic product should contain a viable cell count higher than 10^6 CFU/g in final product which is beneficial to consumer health.

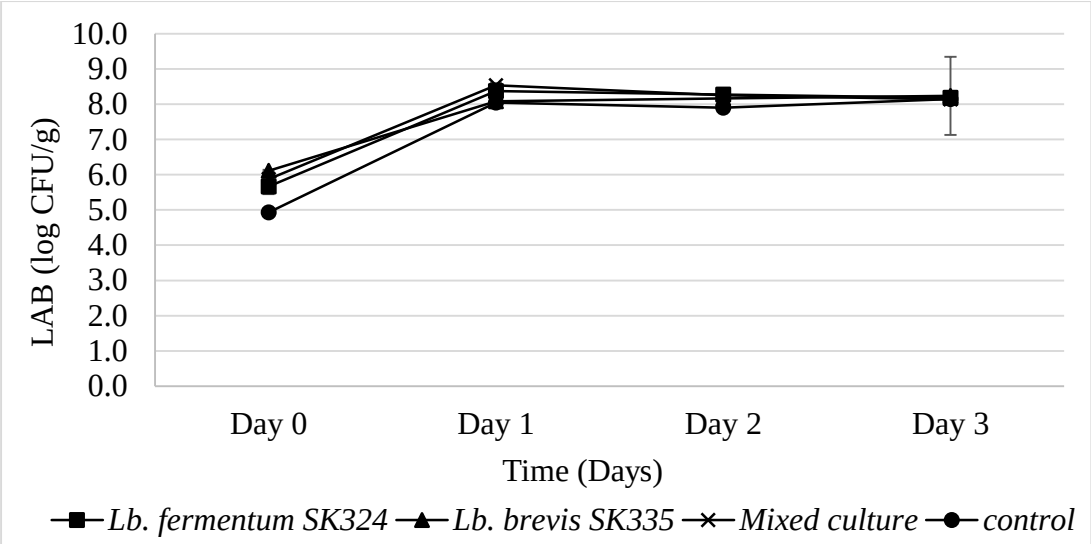


Figure **7** Lactic acid bacteria (LAB) count of fermented Pak-Sian

4.4.3 Total plate count (TPC)

Fermented Pak-Sian subjected to 4 treatments were analyzed for total plate count (TPC). The results showed that TPC tended to slightly increase with fermentation time of 3 days (Figure 8) and the TPC value ranged between 8.16-7.73 log CFU/g. However, the total plate count of fermented Pak-Sian had higher than the Thai- FDA standard which is specified not more than 10^6 CFU/g (The community product standard 1213/2549). This study showed that LAB count increased and was consistent with TPC count, indicating that the use as starter culture may be due to the majority of bacteria growing on the PCA plate which was consistent with the results of Oguntoyinbo *et al*. (2016). This study revealed that the initial TPC count was higher than the LAB count probably due to microbial contamination from the raw material. Sarvan *et al*. (2013) investigated the microbiological changes occurring in blanched cabbage inoculated with *Lactobacillus paracasei* LMG during fermentation and found the presence of a total count but absence of LAB. The detection of total count value can be attributed to the growth of spore-forming bacteria which survive despite the blanching process. Sánchez *et al*. (2001) studied the usage of *Lactobacilli* for Spanish-style green olive fermentation, the treatment with LAB inoculation indicated that the population of viable lactobacilli was higher than the uninoculated

starter culture and inoculation reduced the population of *Enterobacteriaceae*, which is responsible for spoilage.

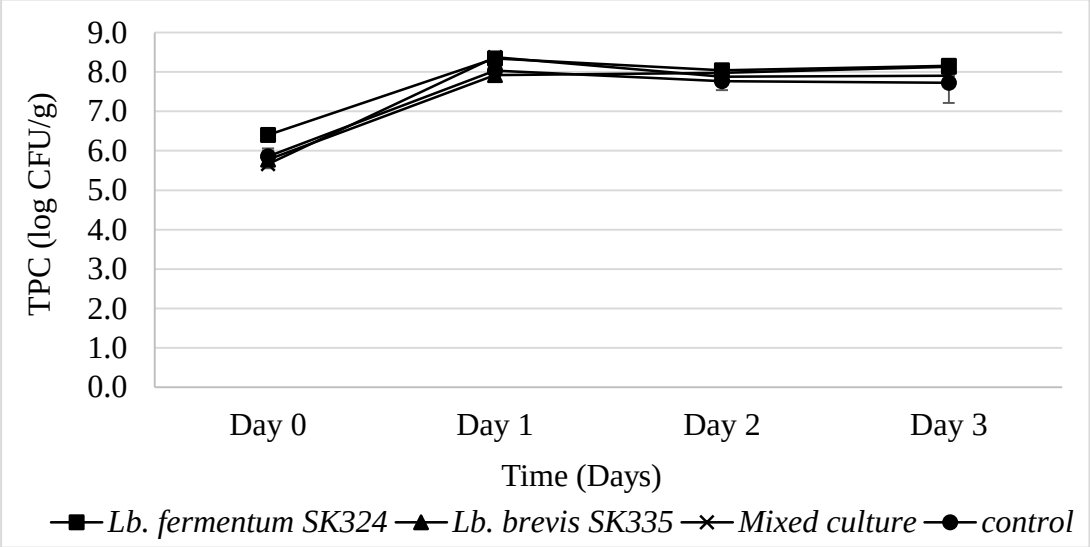


Figure **8** Total plate count of fermented Pak-Sian

4.4.4 Short chain fatty acid (SCFA)

The results of short chain fatty (acetic acid, propionic acid and butyric acid) in fermented Pak-Sian in this study did not exhibit presence of propionic acid and butyric acid whereas acetic acid was found increase. The results of acetic acid production in fermented Pak-Sian using different cultures are shown in Table 26. Increasing fermentation time led to an increase in the concentration of acetic acid in all treatments. A comparison between the acetic acid production in the different treatments showed that *Lb. fermentum* SK324 produced the highest acetic acid followed by *Lb. brevis* SK335. Carbohydrates are digested in the colon that are fermented by anaerobic bacteria and produce short chain fatty acid. These acids are absorbed in the epithelial cell of colon and stimulates the absorption of sodium and water which can affect the growth of the epithelial cell (Scheppach, 1994). Probiotic LAB play an important role by fermenting carbohydrate consisting of short chain fatty acids which contain acetate, propionate and butyrate. They are beneficial health of consumer as they can produce anti-carcinogenic substance which have anti-cancer properties (Kahouli *et al*., 2015). Cummings, Macfarlane, and Englyst (2001) reported short chain fatty acid from LAB contributing to the beneficial health of the consumer

by causing the intestines to become acidic which is suitable for the growth of bacteria and thereby resulting in reduced pathogenic bacteria (Blaut, 2002). Many researches have found that short chain fatty acid have ability to inhibit cancer cells. Thirabunyanon and Hongwittayakorn (2013) found probiotic bacteria isolated from human origin can attach to cancer cell and stimulate the production of short chain fatty acids; butyric and propionic acid which can inhibit the propagation of colon cancer cell. Kahouli *et al*. (2015) reported *Lactobacillus fermentum* can produce short chain fatty acid such as acetic, propionic, and butyric acid, they can anti-proliferative properties inhibit Caco-2 colon cancer cell. However, this experimental found only acetic acid which may be benefit for health. Topping (1996) reported acetate is short chain fatty acid stimulates the reduction of resistance vessels in the colonic vasculature, a change which assists in the maintenance of flow of blood to the liver.

Table ***26*** The Short chain fatty acid production of fermented Pak-Sian

Day	Treatments	Acetic acid (mM)	Propionic acid (mM)	Butyric acid (mM)
0	*L. fermentum* SK324	2.25±0.03^{b}	ND	ND
	L. brevis SK335	2.28±0.01^{b}	ND	ND
	Mixed culture	4.18±0.23^{a}	ND	ND
	Control	2.20±0.01^{b}	ND	ND
1	*L. fermentum* SK324	10.50±0.03^{a}	ND	ND
	L. brevis SK335	9.79±0.08^{b}	ND	ND
	Mixed culture	8.41±0.02^{d}	ND	ND
	Control	8.85±0.02^{c}	ND	ND
2	*L. fermentum* SK324	14.13±0.06^{a}	ND	ND
	L. brevis SK335	11.91±0.03^{d}	ND	ND
	Mixed culture	12.88±0.20^{b}	ND	ND
	Control	12.48±0.02^{c}	ND	ND
3	*L. fermentum* SK324	20.44±0.03^{a}	ND	ND
	L. brevis SK335	13.81±0.03^{c}	ND	ND
	Mixed culture	15.87±0.38^{b}	ND	ND
	Control	12.59±0.30^{d}	ND	ND

Different letters within a column were significantly different ($P \leq 0.05$)

ND; Not detect

4.4.5 Sensory evaluation

Sensory evaluation of fermented Pak-Sian was performed at the end of fermentation by using a 9-point Hedonic Scale and 30 non-trained panelists. The panelist consisted of 19 females and 11 males, the age ranged 21-50 years old, 7 civil servants, 13 students and 10 housekeepers. The sensory evaluation of fermented Pak-Sian using different starter cultures were evaluated based on different attributes such as colour, smell, taste, sourness, texture and overall acceptance (Table 27). The results show that all fermented Pak-Sian treatments had no significant differences on the colour, smell, taste, texture and overall acceptance whereas the sourness attribute was significantly different ($P \leq 0.05$) with the highest score recorded of control (7.56) and no difference with the other treatments. In this study, the panelists were unable to distinguish any differences excepted sourness may be due to the addition of starter culture which resulted in lower pH than control treatment cause sourer taste than control. Thus, panelists may not like the sour taste pose for having a score sour of starter culture lower than the control treatment. The liking score of sourness was about 7-7.56 score (moderately score). In the present study, panelists preferred the control treatment (no addition of starter) rather than starter and mixed culture. This may be attributed to the fact that the control fermentation may contain a variety of bacteria that might contribute to the improvement of unique characteristics of the fermented product (W. Holzapfel, 2002). Fermentation can improve the organoleptic properties of food. During fermentation, LAB can produce flavor metabolites such as diacetyl and organic acid which improve the taste of fermented products and the organic acids can interact with other substances (alcohols and aldehydes) resulting in the production of additional flavor compounds (Bourdichon *et al*., 2012; Liu *et al*., 2011). The cabbage fermented with *Leuconostoc mesenteroides*, displays a firm texture and reduces off-flavors in the product (Johanningsmeier *et al*., 2007). The overall acceptance was finally considered and it was found that the panelists accepted the fermented Pak-Sian and it's score was not different when compared to other treatments. The highest score was conferred to control followed by the mixed culture, *Lb. brevis* SK335 and *Lb. fermentum* SK324, respectively. However, statistical analysis was not significant between control, starter culture and mixed starter culture which indicate the panelist could not detect difference in terms of colour, smell, taste,

texture, and overall acceptance. Sobowale, Olurin, and Oyewole (2007) evaluated the sensory evaluation of lactic acid starter culture fermented cassava and fufu in comparison with traditional starter culture, which revealed that fermented cassava and fufu flavors was not significantly different in terms of colour, odor and texture. Whereas, fermented cassava and fufu product fermented by starter culture had the highest overall acceptance. From this experiment it was observed that the use of starter culture; *Lb. brevis* SK335, *Lb. fermentum* SK324 and mixed starter culture can be used in the production of fermented Pak-Sian. However, the consideration of mixed starter culture for the production of fermented Pak-Sian may increase the chance of cell adhesion in the intestine. W. Holzapfel (2002) and Viander *et al*. (2003) reported the following reasons for the consideration of suitable starter cultures; the ability to reduce fermentation time, improve sensory attributes and probiotic properties, improve safety and reduce the amount of undesirable microorganism.

*Table **27*** Sensory evaluation of fermented Pak-Sian by using different culture (Mean±SD)

	Attributes of sensory evaluation					
Treatments	colour^{ns}	smell^{ns}	taste^{ns}	sour	texture^{ns}	overall acceptance^{ns}
Lb. fermentum SK324	7.50±1.00	7.20±1.18	7.10±0.60	7.10±0.71^{a}	7.36±1.12	7.16±0.59
Lb. brevis SK335	7.33±1.06	7.23±1.19	7.20±0.76	7.00±0.87^{a}	7.30±1.14	7.20±0.61
Mixed culture	7.43±1.00	7.33±1.12	7.13±1.13	7.13±0.81^{a}	7.16±1.11	7.23±0.67
control	7.53±0.89	7.23±1.07	7.33±1.12	7.56±1.07^{ab}	7.23±1.07	7.26±0.90

Difference lowercase in the column within a column was significantly different ($P \leq 0.05$) whereas ns refers not significantly different

CHAPTER V
CONCLUSION

The present study consists of 3 parts; Part I: isolation of LAB from fermented Pak-Sian, Part II: Study on probiotic properties of LAB isolated from fermented Pak-Sian, and Part III: The application of LAB as starter culture in Pak-Sian fermentation.

Part I: Isolation of LAB from fermented Pak-Sian.

For the isolation of lactic acid bacteria (LAB) from fermented Pak-Sian, fermented Pak-Sian was collected from the local markets of the following 4 provinces: Kalasin, Sakon Nakhon, Maha Sarakham and Khon Kaen. From the study, LAB can be selected from 234 presumptive LAB isolates. These presumptive LAB were grouped by whole-cell protein patterns using SDS-PAGE. The results found 61 presumptive LAB species from 8 local markets targeted in this study. These LAB were confirmed and identified by 16S rDNA analysis. We found 17 strains were *Pediococcus pentosaceus* (KS12 KS218 KS230 SK337 MK74), *Pediococcus* sp. KS215, *Lactobacillus plantarum* (SK321 KK53 KK518 MK711 MK724), *Lb. brevis* SK335, *Lb. fermentum* (SK324 SK48 SK434), *Weissella cibraria* (SK415 SK432).

Part II: Study on probiotic properties of LAB isolated from fermented Pak-Sian

The tests carried out to elucidate the probiotic properties of 17 LAB strains include; bile salt tolerance (0.3% bile salt), pH tolerance (pH 2.5), survival in simulated gastric and intestinal tract, antimicrobial activity and antibiotic susceptibility, biogenic amine production and haemolytic activity. This study found that out of 17 LAB strains, only 3 species did not tolerate low pH 2.5 such as *Lb. fermentum* (SK48) and *W. cibraria* (SK415 SK432). Fourteen strains were studied for resistance to bile salt. This experiments showed that 14 strains are resistant to bile salt. The survival rates of the 14 strains were in the range of 65.59-95.13%. The survival capability in simulated gastric and intestinal tract was studied. The results showed that 14 strains were able to survive in simulated gastric and intestinal tract. The survival rates of the 14 strains in simulated gastric were in the range of 16.56-81.45% and 86.16-98.23%, respectively. When 14 strains were investigated for antibiotic susceptibility utilizing (streptomycin, rifampicin, vancomycin, ampicillin,

azithromycin and chloramphenicol) it was found that all 14 species of LAB were resistant to antibiotics except some species such as *Lb. plantarum SK321 KK58, Lb. fermentum SK324. Lb. brevis SK335* that showed moderate susceptibility to cholamphenical and *Lb. fermentum SK434, Lb. plantarum KK53, KK518, MK724* were moderately susceptible to rifampicin. Meanwhile, *Lb. plantarum* SK321, *Lb. fermentum* SK434 *and Lb. plantarum* MK711, MK724 were susceptibility to ampicillin. The activity of LAB was detected by carrying out using agar spot technique and pathogenic bacteria indicators such as *E. coli, S. typhimurium, B. cereus* and *S. aureus*. The results showed that 14 species inhibited all pathogenic bacterial indicators. Most of the species showed effective inhibition against *E. coli*. Thereafter, 14 strains were examined for consumer safety properties such as presence of biogenic amine production and haemolytic activity. The results showed that 14 LAB species did not synthesize biogenic compound and only 8 LAB species showed γ-haemolysis (no haemolysis) such as *P. pentosaceus* (KS12 KS230 SK337 MK74), *Pediococcus* sp. KS215, *Lb. fermentum* (SK 324 SK434) and *Lb. brevis* SK335. Subsequently, it can be extrapolated that all 8 strains of bacteria are safe for consumers. Therefore, only these 8 strains were further evaluated for adhesion capacity. All 8 species had adhesion values of approximately 0-2.39% and it was found that *P. pentosaceus* KS12 and SK335 do not have adhesion capacity, *Lb. fermentum* SK324 and *Lb. brevis* SK 335 showed high percentage of adhesion capacity (2.39% and 2.34%, respectively). Therefore, considering the adhesion properties as the main criteria and ensuring that all of strains have probiotic properties, this study selected *Lb. fermentum* SK324 and *Lb. brevis* SK 335 to be used as probiotic starter culture for the fermentation of fermented Pak-Sian.

Part III: The application of LAB as starter culture in Pak-Sian fermentation.

This study selected 2 strains; *Lb. fermentum* SK324 and *Lb. brevis* SK 335 to be used as probiotic starter culture. This study is divided into 4 treatments; *Lb. fermentum* SK324, *Lb. brevis* SK 335, mixed culture (*Lb. fermentum* SK324, *Lb. brevis* SK 335; 1:1) and control (no addition of starter culture). All 4 treatments were utilized in fermenting of Pak-Sian. The sampling of fermented Pak-Sian was done on the days 0, 1, 2 and 3. Fermented Pak-Sian was analyzed for the following: pH,

tritrable acidity (as %lactic acid), LAB count, total plate count (TPC count) and short chain fatty acid.

The pH of initial fermented Pak-Sian was 6.01-6.09 and on the last day of fermentation was 4.18-4.30. This study found that fermentation using starter cultures (*Lb. fermentum* SK324, *Lb. brevis* SK 335, mixed culture) display a decreasing trend of pH and the pH value was lower than control treatment.

In the study of LAB and TPC count, it was found that LAB and TPC count tended to increase. On the last day of fermentation all treatment had a cell count of 8.13-8.23 log CFU/g and 7.73-8.16 log CFU/g, respectively. Which the value of LAB count were over 10^6 CFU/g that advantage for consumer health. Meanwhile in the study to detect presence of short chain fatty acids in fermented Pak-Sian such as acetic acid, propionic acid and butyric acid, acetic acid production was detected in fermented Pak-Sian while propionic acid and butyric acid were not found. Increase of fermentation time led to a corresponding increase in the concentration of acetic acid in all treatments. On the last day of fermentation, the acetic acid value was 12.59-20.44 mM. The acetic acid value of fermented Pak-Sian to be used as starter culture was higher than the control treatment.

On the 3rd day of fermentation, 4 treatments of fermented Pak-Sian were subjected to a sensory evaluation to evaluate colour, smell, taste, sour texture and overall acceptance. The treatment that gained the panelist's acceptance and was not different from the control treatment was selected. From the experiment, it can be seen that using of probiotic starter culture (*Lb. fermentum* SK324, *Lb. brevis* SK 335, mixed culture) can be feasible in the production of fermented Pak-Sian. This data shows benefit to be used as starter culture to make the better health-promoting fermented Pak-Sian product for consumers with consistent quality due to controlled fermentation, consumer safety, desirable sensory attributes and abundant with probiotic bacteria that are beneficial to human health.

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APPENDICES

APPENDICES A

Chemical and Analysis

1. Salt content (NaCl)

The sample (10 g) was mixed with a small amount of boiled DI water or preheated in the water bath and then the volume was adjusted to 100 mL in volumetric flask using DI water. The mixture was filtered using a Whatman no.4 filter paper and 10 mL of filtrate was pipetted into an Erlenmeyer flask. The sample was titrated against 0.1 M $AgNO_3$ and 1 ml of 5% K_2CrO_4 was used as an indicator till the equivalence point was expressed as an orange/red brick colour. The content of NaCl was calculated as % acid equivalents.

% Salt (NaCl) = [(A x B x C) x 0.005844 x1000] / D x E

whereas A = the total volume of 0.1 N $AgNO_3$ used for titration (mL)

B = volume made up (mL)

C = the concentration of $AgNO_3$ used

D = weight (g, mL) of sample

E = volume of sample use for titration (mL)

2. Denaturing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The protein sample was determined by using SDS-PAGE according to Laemmelli (1970). 20 µg of protein sample and 2x SDS-PAGE loading buffer was mixed and then boiled at 100 °C for 2 min before resolving on a 12.5% SDS-PAGE gel in 1x SDS running buffer by using Mini PROPEIN Tetra cell apparatus (Bio-Rad, UK). The gel was subjected to electrophoresis for 50 min at 180 V before staining with Coomassie Brilliant Blue R-250 staining solution for 15 min. After staining, the gel was washed with DI water before destaining again with destain solution until the background of gel was clear. Finally, the band of protein was visible.

2.1 The composition preparation

2.1.1 Preparation of 12.5% SDS-PAGE for 2 gels

Preparation of 12.5% separating gel for 10 mL by mixing of 3.3 mL of Milli-Q water, 4.0 mL of 30% (w/v) acrylamide: bis-acrylamide stock solution (0.8%) in the ratio (37.5:1), 2.5 mL of 1.5 M Tris-HCl (pH 8.8), 100 µL of 10% SDS,

100 µL of ammonium persulfate and 4 µL of N, N, N', N'-Tetramethylethylenediamine (TEMED).

2.1.2 Preparation of 4% stacking gel for 3 mL

A 2.1 mL of Milli-Q water, 0.5 mL of 30% (w/v) acrylamide with 0.8% bis-acrylamide stock solution in the ratio (37.5:1), 0.38 mL of 1.0 M Tris-Cl (pH 6.8), 30 µL of 10% SDS, 30 µL of ammonium persulfate (APS) and 3 µL of N, N, N', N'-Tetramethylethylenediamine (TEMED) were mixed together.

2.2 Preparation of SDS-PAGE loading buffer 2x

A 0.5 M of Tris-HCl (pH 6.8), 4.4% (v/v) of SDS, 20% (v/v) of glycerol (GE healthcare), 2% (v/v) of 2-mercaptoethanol and 0.05% of bromophenol blue were dissolved in Milli-Q water.

2.3 Preparation of 10x SDS Running Buffer for 1 L

A 30 g of Tris-base, 144 g of glycine and 10 g of SDS were dissolved in Milli-Q water.

2.4 Preparation of Coomassie staining solution for 1 L

A 2.5 g of Coomassie blue R-250, 450 mL of methanol, 450 mL of Milli-Q water and 100 mL of acetic acid were mixed.

2.5 Destaining solution for 1 L

A 300 mL of methanol, 100 mL of acetic acid, 600 mL of Milli-Q water and 100 mL of acetic acid.

3. Buffer preparation

4.1 Preparation of citrate buffer (pH 4.0)

To prepare 100 mL of 0.1 M citrate buffer at pH 4.0, the solution was mixed between the 59 mL of 0.1 M citric acid monohydrate ($C_6H_8O_7.H_2O$) (solution A) and 41 mL of 0.1 M trisodium citrate, dehydrate ($C_6H_5O_7Na_3.2H_2O$) (solution B).

4.2 Preparation of phosphate buffer (pH 7.8)

To prepare 100 mL of 0.1 M phosphate buffer at pH 4.0, the solution was mixed between the 45.75 mL of 0.2 M sodium phosphate, dibasic dihydrate ($Na_2HPO_4.2H_2O$) (solution A) and 4.25 mL of 0.2 M sodium phosphate monobasic monohydrate ($NaH_2PO_4.H_2O$) (solution B).

4. Mucin purification

The purified porcine gastric mucin was added in 25 mL of 0.1 M NaCl containing 0.02 M phosphate buffer (pH 7.8) with a few drops of toluene before stirring for 24 h. After stirring for 1 h, the mixture was adjusted to pH 7.2 with 2 N NaOH and centrifuge was carried out at 10,000g for 10 min. The supernatant was collected and cooled down until a temperature of 0±2 °C was reached and then 60% (v/v) of ethanol was added to precipitate the mucin. The sediment was suspended twice with 0.1 M NaCl and precipitated with 60% ethanol. The sediment was washed with ethanol and conducted to dialysis using Milli-Q water for 24 h. Dialyzed mucin was dried using freeze dryer and dried mucin was stored at -20 °C prior use.

5. Starter culture preparation for Pak-Sian fermentation

The bacteria strains were cultivated in MRS broth for 24 h. A 100 µL of bacterial strains were inoculated into 3 mL of MRS broth and incubated for 18 h at 37 °C. Afterward, the bacteria cultures were centrifuged at 12,000g, 4 °C for 5 min. The supernatants were discarded and the collected sediment was washed 3 or 4 times with 0.85% NaCl. The sediment in 0.85% NaCl was measured to obtain the optical density (OD) at 0.1 which represent the bacteria concentration of 10^6 CFU/mL.

6. Preparation of gastric solution

The gastric solution was prepared by mixing 0.5 g NaCl with 0.3 g pepsin. The mixture was adjusted to pH 3 and the volume was adjusted up to 100 mL with DI water followed by filtering through a nylon membrane (0.45 µm).

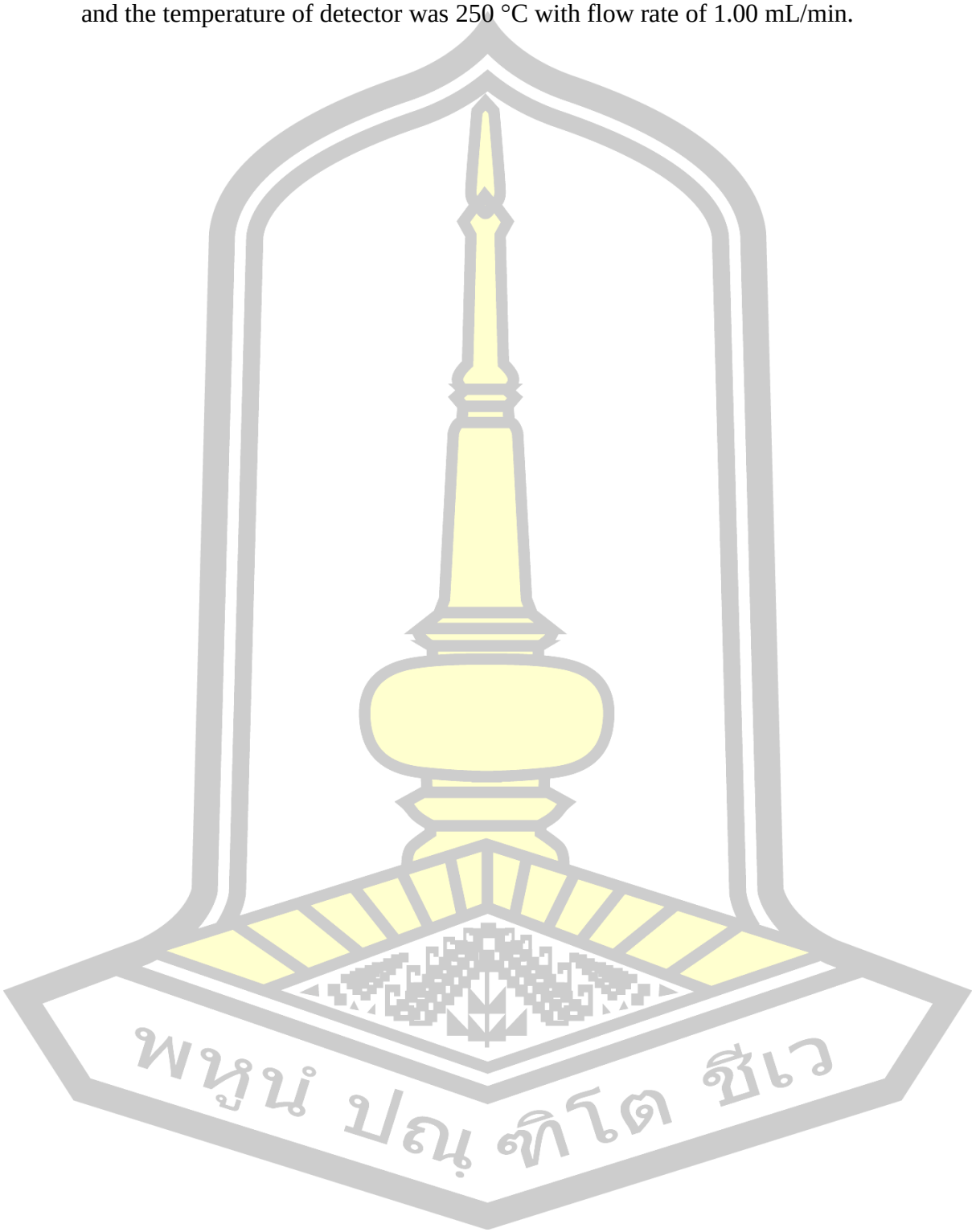
7. Preparation of intestinal juice

Intestinal juice comprising of a mix between 0.1 g pancreatin and 0.5 g NaCl was adjusted to pH 6 and the final volume was adjusted to 100 mL with DI water and filtered through a 0.45 µm nylon membrane.

8. Identification and quantify of lactic acid and short chain fatty acid

Cell suspension of fermented Pak-Sian was filtered by using a 0.45 µm nylon membrane and the short chain fatty acid included acetic acid, propionic acid and butyric acid was determined by using gas chromatography (GC Varian CP-3800 FID-Detector), column DB-FFAP (30m x 0.25mm x 0.25um), Agilent technologies USA. The initial temperature of the column was 80 °C, held for 5 min and then increased to 170 °C at a (10 °C/min) rate and held for 0 min, then increased to 250 °C at the rate of

(30 °C/min) and held for 5 min. The temperature of injector was 250 °C, split 30:1 and the temperature of detector was 250 °C with flow rate of 1.00 mL/min.



APPENDICES B

Culture media and Reagents

1. MRS (de Man Rogosa and sharpe) broth

Bacto peptone (10.0 g), bacto beef extract (10.0 g), bacto yeast extract (5.0g), glucose (20.0 g), sorbitan monoleate complex (10.0 g), ammonium citrate (2.0 g), sodium acetate (5.0 g), magnesium sulfate (0.2 g), manganese sulfate (0.05 g), potassium phosphate, dibasic (2.0 g) were mixed in distilled water (1,000 ml). The mixture was adjusted the pH at 6.5 and was then autoclaved at 121 °C, 15 psi for 15 min.

2. MRS (de Man Rogosa and sharpe) agar

MRS agar was prepared by mixing of bacto peptone (10.0 g), bacto beef extract (10.0 g), bacto yeast extract (5.0g), glucose (20.0 g), sorbitan monoleate complex (10.0 g), ammonium citrate (2.0 g), sodium acetate (5.0 g), magnesium sulfate (0.2 g), manganese sulfate (0.05 g), potassium phosphate, dibasic (2.0 g), agar (15.0 g) and distilled water (1,000 ml). The mixture was adjusted the pH at 6.5 and was then autoclaved at 121 °C, 15 psi for 15 min.

3. Nutrient agar (NA)

Nutrient agar was prepared by beef extract (3.0 g), peptone (5.0 g), agar (15.0 g), distilled water (1,000 ml), pH 7.2-7.4. The mixture was autoclaved at 121 °C, 15 psi for 15 min.

4. PCA (Plate Count Agar)

Pancreatic digest of casein (5.0g), yeast extract (2.5g) dextrose (1.0g), agar (15.0g) were mixed in distilled water (1,000 ml) at pH 7.0 and then autoclaved at 121 ºC, 15 psi for 15 min.

5. Catalase testing (3% H_2O_2)

The reagent was prepared by 35% H_2O_2 (8.6 ml) mixed in distilled water (1,000 ml) and stored in refrigerator prior used.

6. Chemical for gram straining

6.1. Crystal violet

Reagent A: dissolve crystal violet (2.0 g) in 95% ethyl alcohol (20 ml).

Reagent B: dissolve ammonium oxalate (0.8 g) in distilled water (80 ml).

The reagent A and B were mixed and incubated for 24 h before pass through the filtered paper. The crystal violet straining reagent was obtained.

6.2. Preparation of 95 % ethyl alcohol

Ethyl alcohol (95 ml) was adjusted in distilled water up to 100 ml. A 95 % ethyl alcohol was obtained and use for decolorizing solvent.

6.3. Gram iodine (mordant)

Mordamt was prepared by iodine (1.0 g) and potassium iodine (2.0 g) were mixed and 300 ml of distilled water was slowly added until the iodine was dissolved and kept in the brown reagent bottle.

6.4. Safranin (counterstain)

Safranin was prepared by safranin O (2.5% w/v) in 95 % ethyl alcohol (10 ml) and 100 ml of distilled water was added.

6.5 Iodine reagent

Iodine reagent was prepared by mixing of iodine (1.0 g) and potassium iodine (20.0 g) in distilled water (100 ml).

APPENDICES C

Acceptance and Consumer Preference Testing of Fermented Pak-Sain Products Using Pure Culture

Instruction: Please check (✓) i☐ that you think it mostly matches with your feelings

Part 1: General information of respondents

1.1 Gender

Female ☐ Male ☐

1.2 Age

☐ 10-20 years ☐ 21-30 years

☐ 31-40 years ☐ 41-50 years

1.3 Occupation

☐ Student ☐ Trading / personal business

☐ Employee ☐ Civil servant / state enterprise

☐ Other (details).....

1.4 Pleased to be participate to sensory evaluation of fermented Pak-Sain products that are fermented using starter culture.

☐ Pleased to be participate ☐ Do not wish to participate

Part 2

Instruction: Please gargle with the given drinking water before evaluate the sample and record your like or dislike score in each attribute that it mostly matches with your feelings, and please gargle before evaluate the next examples.

Sensory Evaluation Form

(9 points hedonic scale)

Product name: Fermented Pak-Sain products using starter culture

Name.....Date.....

Instruction: Please evaluate the given samples by following the code in the table from the left to the right side, then recode your like or dislike score as;

9 = like extremely 8 = like very much 7 = like moderately 6 = like slightly 5 = neither like nor dislike4 = dislike slightly 3 = dislike moderately 2 = dislike very much 1 = dislike extremely

(Please gargle between the examples)

Attributes /Code	Code	Code	Code	Code	Code	Code	Code	Code .....
Color								
Flavor								
Taste								
Sour								
Texture								
Overall liking								

Suggestion

……

……

……

……..

…….. Thank you very much……

APPENDICES D

Picture of Experiment

*Figure **9*** Purified LAB by streaking on MRS+0.05%BCP

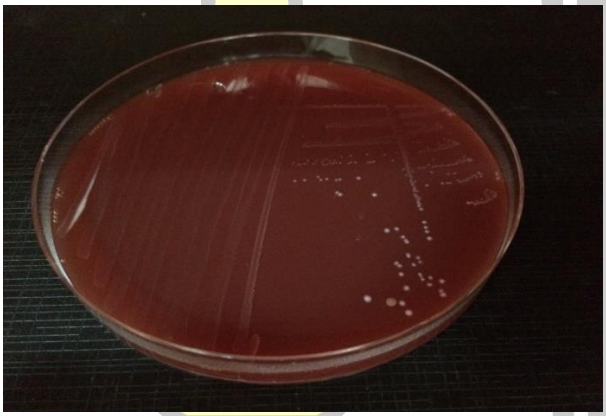


*Figure **10*** Haemolytic activites of LAB (no haemolysis)

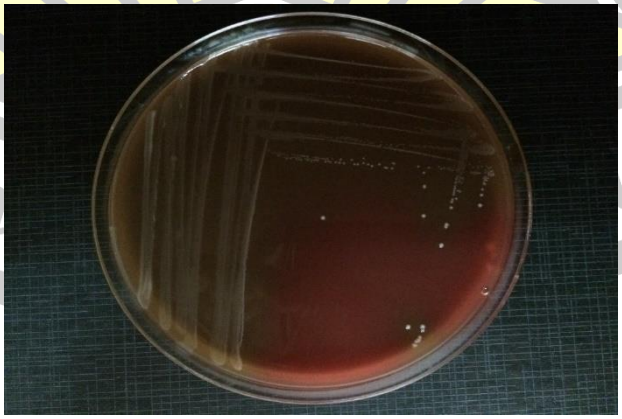


*Figure **11*** Haemolytic activites of LAB (γ- haemolysis)

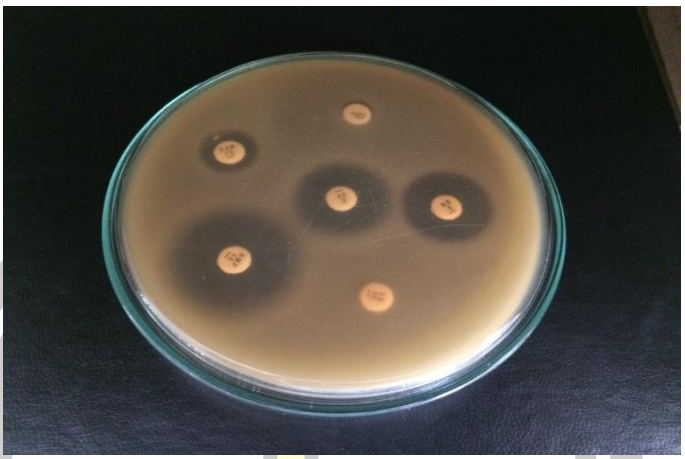


*Figure **12*** Antibiotic activity of LAB

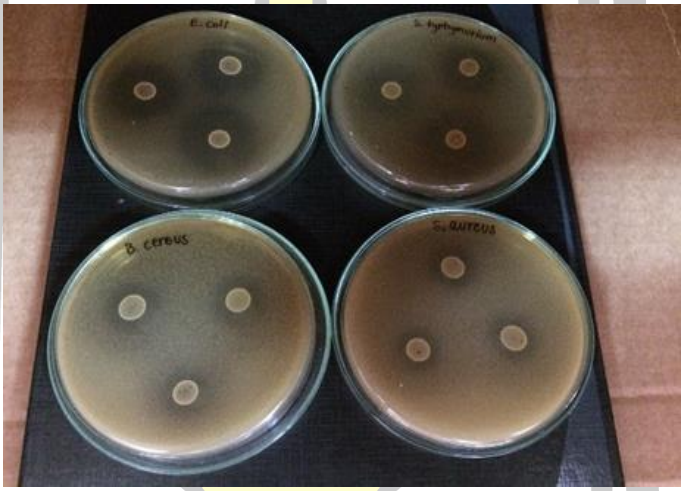


*Figure **13*** Antimicrobial activity of LAB by agar spot test method

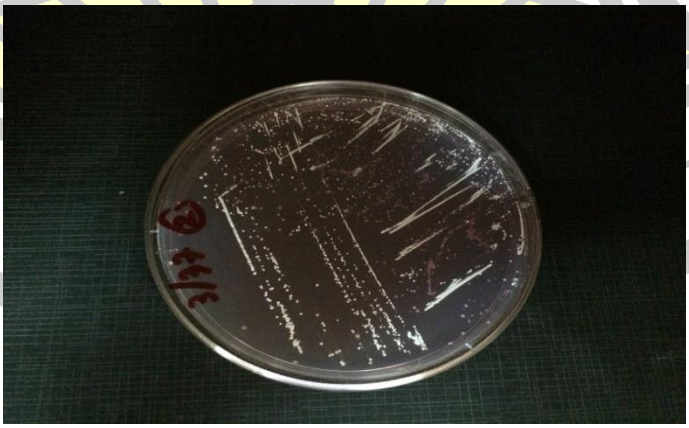


*Figure **14*** Biogenic amine activity of LAB (no biogenic amine activity)

APPENDICES E

SDS PAGE Analysis

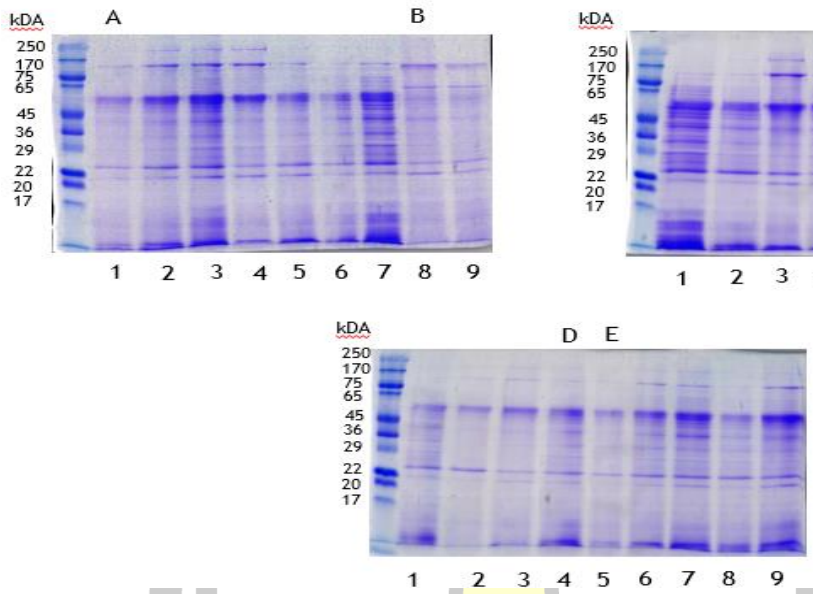


*Figure **15*** The whole-cell protein patterns of presumptive LAB isolates on SDS-PAGE (Kalasin; KS1)

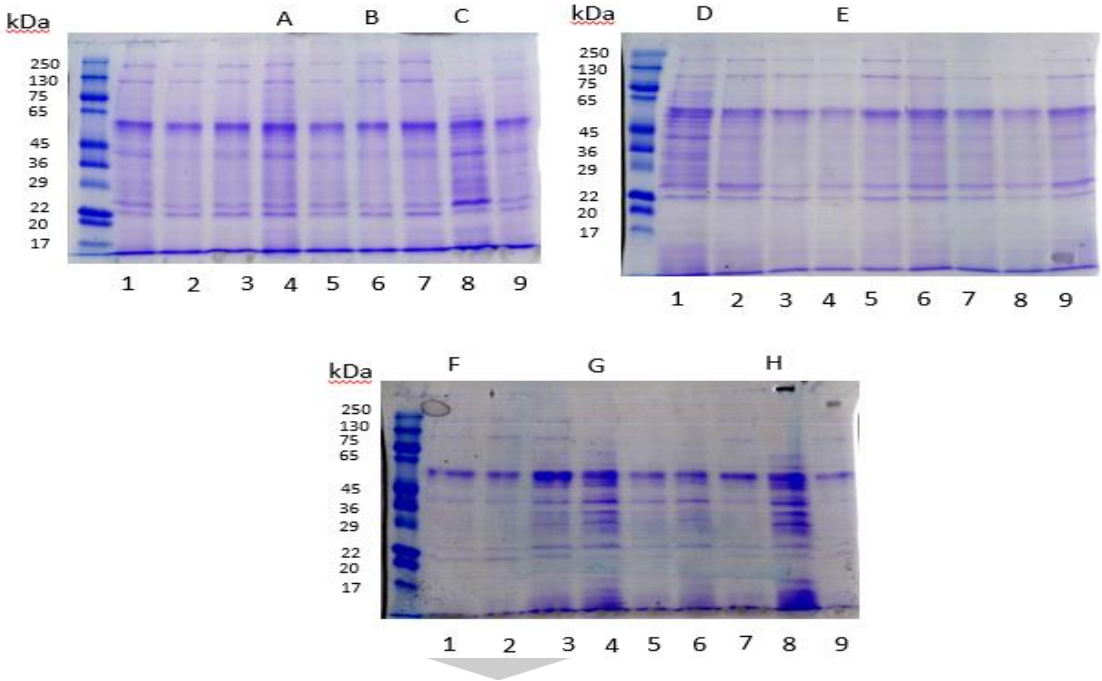


*Figure **16*** The whole-cell protein patterns of presumptive LAB isolates on SDS-PAGE (Kalasin; KS2)

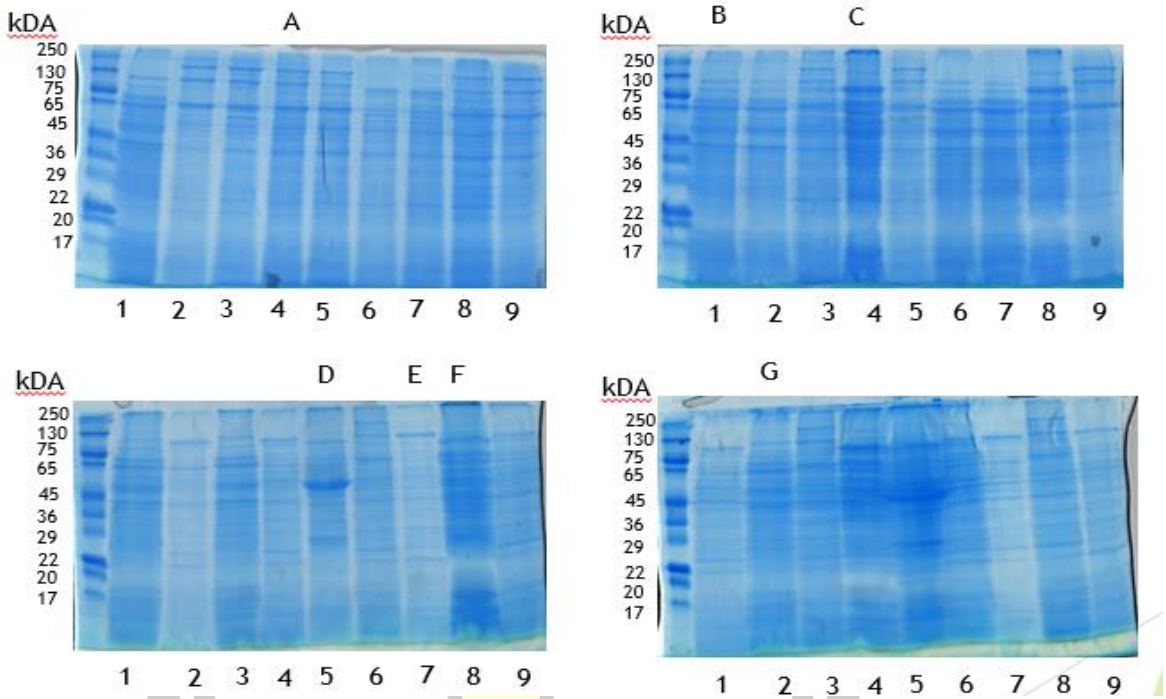


*Figure **17*** The whole-cell protein patterns of presumptive LAB isolates on SDS-PAGE (Sakonnakhon; SK1)

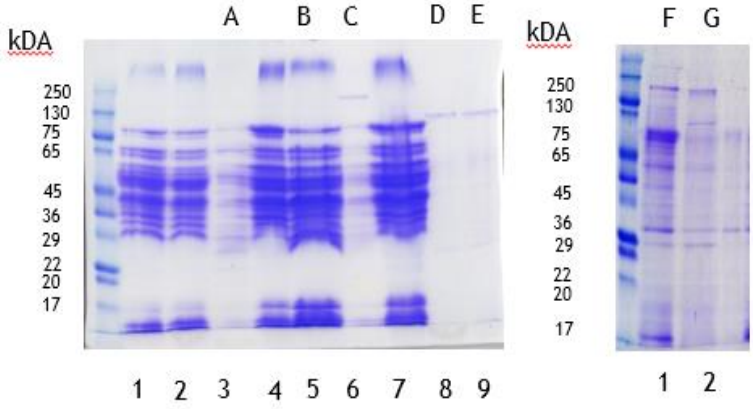


*Figure **18*** The whole-cell protein patterns of presumptive LAB isolates on SDS-PAGE (Sakonnakhon; SK2)

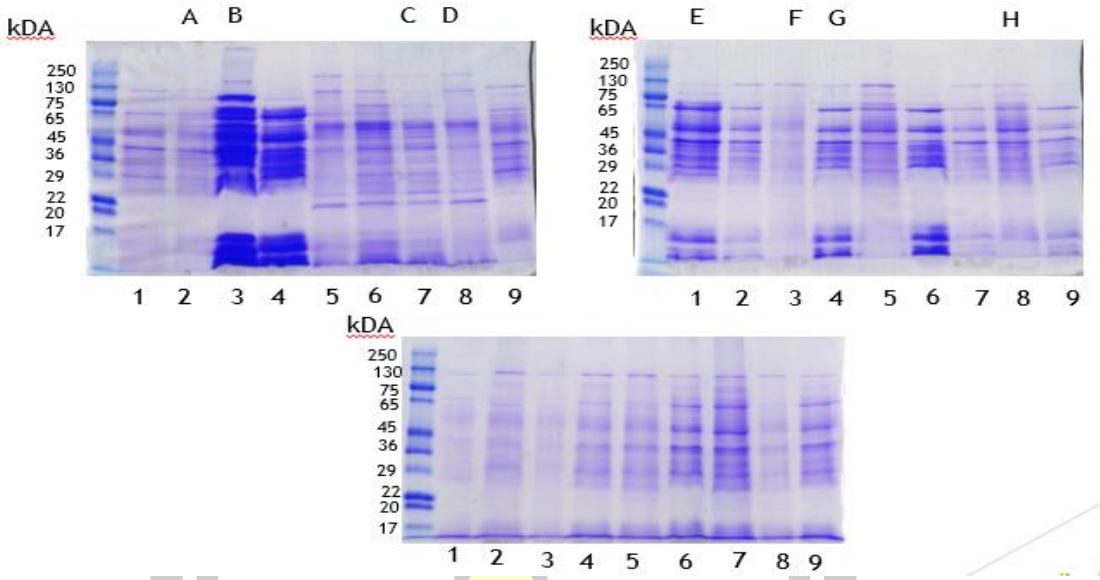


*Figure **19*** The whole-cell protein patterns of presumptive LAB isolates on SDS-PAGE (Khonkean; KK1)

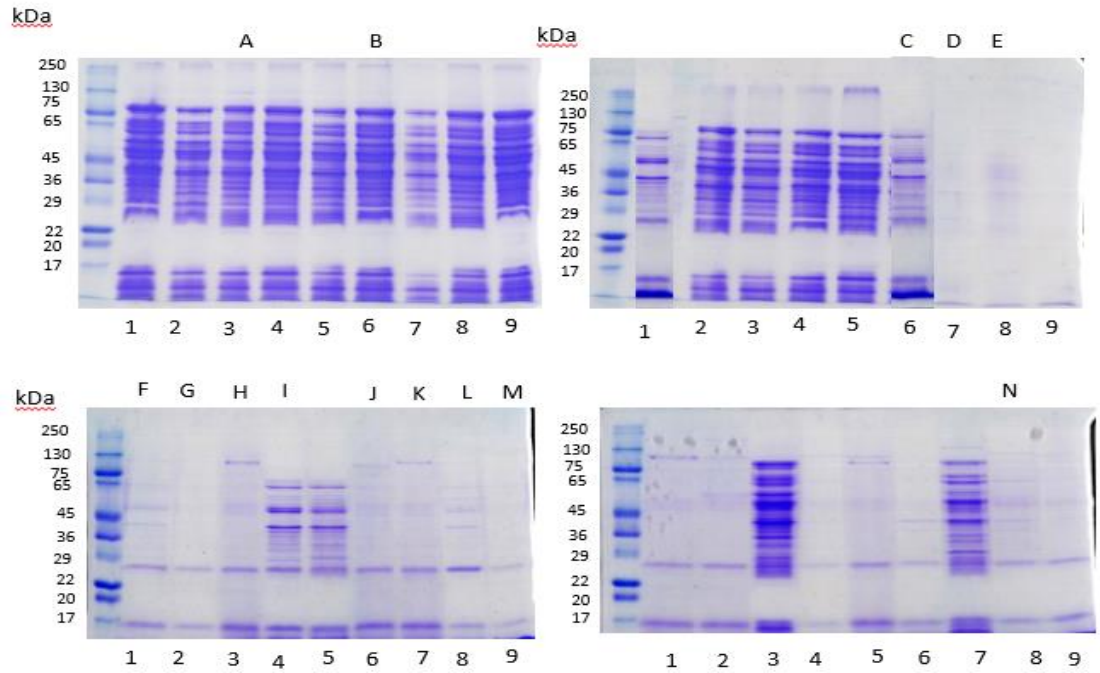


*Figure **20*** The whole-cell protein patterns of presumptive LAB isolates on SDS-PAGE (Khonkean; KK2*)*

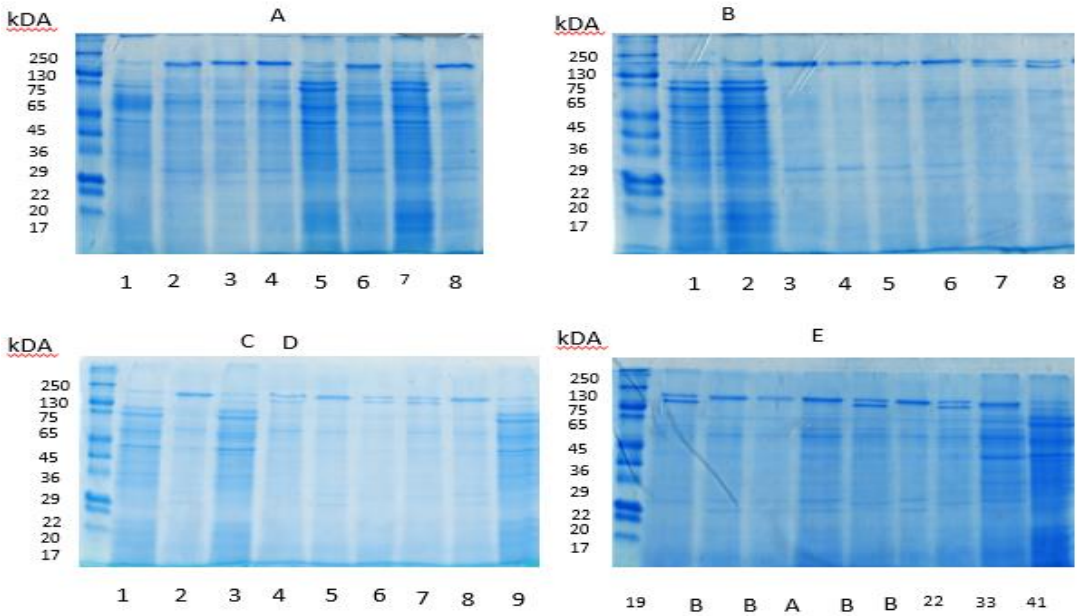


*Figure **21*** The whole-cell protein patterns of presumptive LAB isolates on SDS-PAGE (Mahasakham; MK1)

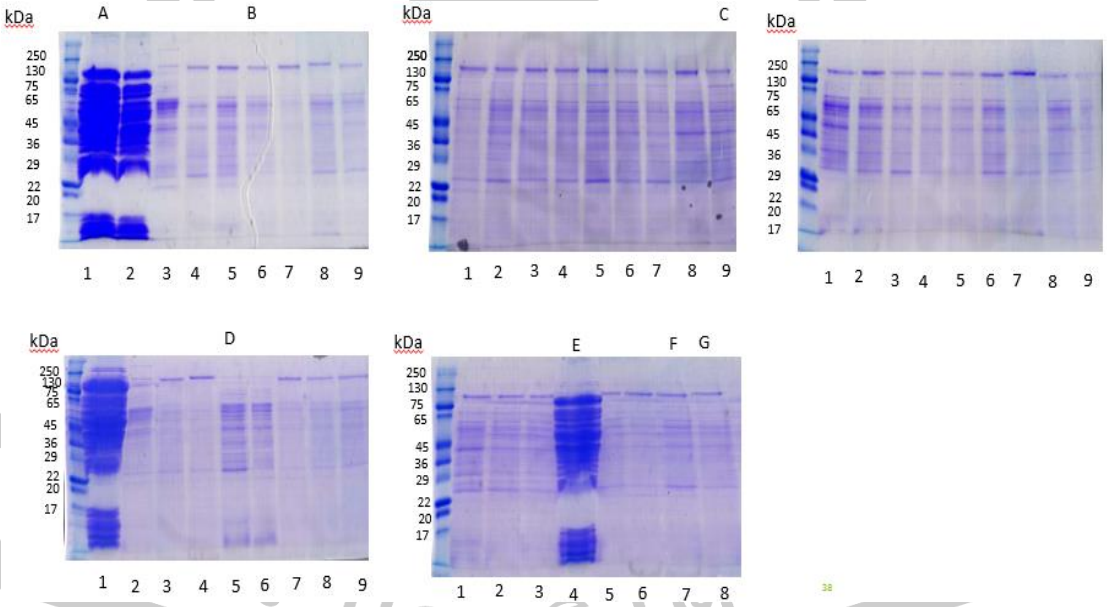


*Figure **22*** The whole-cell protein patterns of presumptive LAB isolates on SDS-PAGE (Mahasakham; MK2

APPENDICES F

List of Probiotic Microorganisms in Foods

Attachment of Notification of the Ministry of Public heath

1. *Bacillus coagulans*
2. *Bifidobactrium adolescentis*
3. *Bifidobacterium animalis*
4. *Bifidobacterium bifidum*
5. *Bifidobacterium breve*
6. *Bifidobacterium infantis*
7. *Bifidobacterium lactis*
8. *Bifidobateriumlongum*
9. *Bifidobacterium pseudolongum*
10. *Enterococcus durans*
11. *Enterococcus faecium*
12. *Lactobacillus acidophilus*
13. *Lactobacillus crispatus*
14. *Lactobacillus gasseri*
15. *Lactobacillus johnsonii*
16. *Lactobacillus paracasei*
17. *Lactobacillus reuteri*
18. *Lactobacillus rhamnosus*
19. *Lactobacillus salivarius*
20. *Lactobacillus zeae*
21. *Propionibacterium arabinosum*
22. *Staphylococcus sciuri*
23. *Saccharomyces cerevisiae subsp. Boulardii*

Reference: Bulletin of the international Dairy Federation No. 377/2002

APPENDICES G

Antibiotic susceptibility

*Table **28*** Diameters for disc diffusion antibiotic susceptibility activity (Charteris *et al*., 1998)

Antibiotic	Disc concentration (µg)	Diameters (mm.)		
		R	MS	S
Ampicillin	10	≤12	13-15	≥16
Vancomycin	30	≤14	15-16	≥17
Streptomycin	10	≤11	12-14	≥15
Choramphenicol	30	≤13	14-17	≥18
Azytromycin	15	≤13	14-17	≥18
Rifampicin	5	≤14	15-17	≥18

BIOGRAPHY

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