ISOLATION AND CHARACTERIZATION OF PROBIOTICS FROM FERMENTED FOODS FOR DEVELOPING SYNBIOTIC POWDER

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เรื่อง : การแยกและศึกษาคุณสมบัติของโพรไบโอติก จากอาหารหมักดองเพื่อการพัฒนาผงซินไบโอติก

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คำสำคัญ : แบคทีเรียกรดแลคติก, โพรไบโอติก, พรีไบโอติก, ซินไบโอติก

แบคทีเรียกรดแลคติกเป็นกลุ่มของโพรไบโอติกที่มีประโยชน์ต่อสุขภาพ การศึกษาพบว่าแบคทีเรียกรดแลคติกสามารถยับยั้งจุลินทรีย์ก่อโรคในระบบทางเดินอาหาร ลดระดับไขมันในเลือด และส่งเสริมการทำงานของระบบภูมิคุ้มกันได้ งานวิจัยนี้มีวัตถุประสงค์เพื่อแยกและจำแนกคุณลักษณะของแบคทีเรียกรดแลคติกจากอาหารหมักดองท้องถิ่น และประเมินคุณสมบัติความเป็นโพรไบโอติกเพื่อนำไปพัฒนาเป็นผงซินไบโอติก โดยนำแบคทีเรียกรดแลคติก จำนวน 83 ไอโซเลต มาคัดกรองฤทธิ์ต้านแบคทีเรียเบื้องต้นด้วยวิธี cylinder plate ต่อแบคทีเรียก่อโรค จำนวน 6 สายพันธุ์ ได้แก่ *Escherichia coli* ATCC 25922, *Salmonella typhimurium* DMST 560, *Pseudomonas aeruginosa* DMST 4739, *Staphylococcus aureus* ATCC 25923**,** *Bacillus cereus* DMST 5040 และ *Salmonella enteritidis* DMST 1567 ทำการคัดเลือกแบคทีเรียกรดแลคติกจำนวน 10 ไอโซเลต ที่แสดงฤทธิ์ในการยับยั้งแบคทีเรียก่อโรคแต่ละสายพันธุ์ได้สูงสุดไปทดสอบความสามารถในการทนต่อสภาวะกรดและเกลือน้ำดี แบคทีเรียกรดแลคติกที่มีศักยภาพในการเป็นโพรไบโอติก จำนวน 3 ไอโซเลต คือ P09, P10 และ P11 สามารถทนต่อสภาวะกรดและเกลือน้ำดีได้สูง จึงถูกคัดเลือกเพื่อนำไปทดสอบฤทธิ์ต้านแบคทีเรียก่อโรค โดยการปรับความเป็นกรดด่างของส่วนใสจากอาหารเพาะเลี้ยงเชื้อให้มีคุณสมบัติเป็นกลาง (pH 6.5) ผลการทดสอบพบว่าหลังจากปรับค่า pH ให้เป็นกลาง แบคทีเรียกรดแลคติกทั้ง 3 ไอโซเลต ไม่แสดงฤทธิ์ในการยับยั้งแบคทีเรียก่อโรค ในขณะที่พบการยับยั้งแบคทีเรียก่อโรคของส่วนใสจากอาหารเพาะเชื้อที่ไม่ได้ปรับความเป็นกรดด่างให้เป็นกลาง ดังนั้นฤทธิ์การยับยั้งแบคทีเรียของทั้ง 3 ไอโซเลตน่าจะเป็นผลเนื่องจากกรดอินทรีย์ที่แบคทีเรียผลิตขึ้นในส่วนใสจากอาหารเพาะเลี้ยงเชื้อ แบคทีเรียทั้ง 3 ไอโซเลต (P09, P10 และ P11) ให้ผลลบกับการทดสอบการทำลายเม็ดเลือดแดงและการทดสอบการสร้างเอนไซม์ดีออกซีไรโบนิวคลีเอส การวิเคราะห์ลำดับนิวคลีโอไทด์ของยีน 16S rDNA สามารถระบุได้ว่าไอโซเลต P09, P10 และ P11 คือ *Lactobacillus plantarum* ผลการทดสอบการเกาะติดเซลล์เยื่อบุผนังลำไส้ ในเซลล์ Caco-2 พบว่า *L. plantarum*

P10 สามารถเกาะติดเซลล์ Caco-2 ได้ดีที่สุด ที่ค่าร้อยละการเกาะติดเท่ากับ 4.52 ดังนั้นแบคทีเรีย *L. plantarum* P10 ถูกนำไปศึกษาฤทธิ์ต้านแบคทีเรีย โดยการบ่มแบคทีเรีย *L. plantarum* P10 ร่วมกับแบคทีเรียก่อโรค ผลการทดสอบพบว่าสายพันธุ์นี้สามารถยับยั้งการเจริญของแบคทีเรียก่อโรค *E. coli* ATCC 25922, *S. typhimurium* DMST 560, *S. aureus* ATCC 25923 และ *B. cereus* DMST 5040 ได้อย่างมีนัยสำคัญทางสถิติ ($p<0.05$) เมื่อบ่มร่วมกันเป็นเวลา 24 ชั่วโมง

นอกจากนี้ *L. plantarum* P10 ถูกนำไปใช้เป็นหัวเชื้อโพรไบโอติกในการพัฒนาเป็นผลิตภัณฑ์ผงซินไบโอติก โดยการเตรียมผงซินไบโอติกด้วยเทคนิคการทำแห้งแบบเยือกแข็ง ซึ่งมีส่วนผสมของ *L. plantarum* P10 ร่วมกับผงอินูลินที่สกัดได้จากหัวแก่นตะวัน และมีนมขาดมันเนยหรือมอลโตเดกซ์ตรินเป็นสารป้องกันเซลล์จากการเกิดผลึกน้ำแข็ง เมื่อนำผงซินไบโอติกไปทดสอบความสามารถในการรอดชีวิตภายใต้สภาวะจำลองของระบบทางเดินอาหาร พบว่า *L. plantarum* P10 มีร้อยละการรอดชีวิตที่สูง อย่างไรก็ตามตำรับผงซินไบโอติกที่ใช้นมขาดมันเนยเป็นองค์ประกอบสามารถทนต่อสภาวะจำลองระบบทางเดินอาหารได้สูงกว่าตำรับที่ใช้มอลโตเดกซ์ตริน อีกทั้งเมื่อเก็บรักษาผงซินไบโอติกที่อุณหภูมิตู้เย็นและที่อุณหภูมิห้อง ตำรับที่เตรียมด้วยนมขาดมันเนยมีความคงตัวที่ดีทั้งด้านการรอดชีวิตของเซลล์โพรไบโอติก (มากกว่า 10^7 CFU/ml) และฤทธิ์ยับยั้งแบคทีเรียก่อโรค อย่างไรก็ตามผงซินไบโอติกยังคงแสดงฤทธิ์ต้านแบคทีเรียก่อโรคทั้ง 4 ชนิด (*E. coli* ATCC 25922, *S. typhimurium* DMST 560, *B. cereus* DMST 5040 และ *S. aureus* ATCC 25923) เมื่อเก็บที่อุณหภูมิตู้เย็นและอุณหภูมิห้อง เป็นเวลา 12 และ 8 สัปดาห์ตามลำดับ ผลการศึกษานี้สรุปให้เห็นว่า *L. plantarum* P10 มีคุณสมบัติที่ดีตามเกณฑ์การคัดเลือกโพรไบโอติก และอาจเหมาะสำหรับนำไปประยุกต์ใช้ในผลิตภัณฑ์ซินไบโอติกที่มีฤทธิ์ต้านจุลินทรีย์ก่อโรคในระบบทางเดินอาหารได้

ABSTRACT

TITLE : ISOLATION AND CHARACTERIZATION OF PROBIOTICS FROM FERMENTED FOODS FOR DEVELOPING SYNBIOTIC POWDER
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KEYWORDS : LACTIC ACID BACTERIA, PROBIOTIC, PREBIOTIC, SYNBIOTIC

Lactic acid bacteria (LAB) are a group of probiotics conferring health benefits. Several studies have reported that LAB can inhibit pathogenic bacteria in the digestive system, reduce blood cholesterol levels, and improve functions of the immune system. The objectives of this study were to isolate and characterize LAB from local fermented foods and to determine the probiotic properties for developing a synbiotic powder. Eighty three LAB isolates were first screened for the antibacterial activity against six pathogenic bacteria: *Escherichia coli* ATCC 25922, *Salmonella typhimurium* DMST 560, *Pseudomonas aeruginosa* DMST 4739, *Staphylococcus aureus* ATCC 25923**,** *Bacillus cereus* DMST 5040*,* and *Salmonella enteritidis* DMST 1567 by the cylinder plate method. Ten LAB isolates showing the highest inhibition zone against each pathogen were further selected for acid and bile salt tolerances. Three potential probiotic strains: P09, P10, and P11 exhibiting acid and bile salt tolerances were evaluated an antimicrobial activity of their culture supernatants after neutralization. Results showed that the neutralized supernatants did not show their antimicrobial activity, while non-neutralized supernatants did. Therefore, the antimicrobial activity of these strains may be due to organic acids presenting in the supernatants. All three isolates (P09, P10 and P11) also showed negative results for haemolytic and DNase tests. The 16S rDNA gene analysis revealed that P09, P10 and P11 isolates were all identified as *Lactobacillus plantarum*. The isolate *L. plantarum* P10 showed the highest adhesion to Caco-2 cells at the level of 4.52%. *L. plantarum* P10 was thus investigated the antibacterial activity

against bacterial pathogens by a co-culture assay. The result demonstrated that the strain had a significantly inhibitory effect on the growth of *E. coli* ATCC 25922, *S. typhimurium* DMST 560, *S. aureus* ATCC 25923 and *B. cereus* DMST 5040 after co-incubating for 24 hours ($p<0.05$).

Additionally, *L. plantarum* P10 served as the probiotic starter for developing its synbiotic powder. The freeze-dried synbiotic powder was prepared by combining the strain with an inulin extracted from Jerusalem Artichoke together with skim milk or maltodextrin as a cryoprotectant. The developed synbiotic powder exhibited a high survivability of *L. plantarum* P10 under the simulated gastrointestinal (GI) conditions. However, the synbiotic powder formulated with skim milk showed a higher GI tolerance than that of maltodextrin. Moreover, the synbiotic powder with skim milk showed a relatively high stability in both its cell viability (more than 10^7 CFU/ml) and antimicrobial activity during storage at refrigerated and room temperatures. However, the antibacterial activity against 4 pathogenic bacteria (*E. coli* ATCC 25922, *S. typhimurium* DMST 560, *B. cereus* DMST 5040 and S. *aureus* ATCC 25923) still remained up to 12 weeks while keeping it refrigerated but only up to 8 weeks at room temperature. In conclusion, the results obtained from this study indicated that *L. plantarum* P10 satisfied the criteria for a potential probiotic and may be suitable for applying the synbiotic product against food-borne pathogens.

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ABBREVIATION AND SYMBOLS

ABBREVIATION AND SYMBOLS	MEAN
CFU	Colony forming unit
CO_2	Carbon dioxide
DNase	Deoxyribonuclease
DNA	Deoxyribonucleic acid
g	Gram
GIT	Gastrointestinal tract
hrs	Hours
JA	Jerusalem artichoke
L	Liter
LAB	Lactic acid bacteria
log	Logarithm
MHA	Mueller Hinton agar
mL	Milliliter
mm	Millimeter
MRS agar	de Man, Rogosa and Sharpe agar
MRS broth	de Man, Rogosa and Sharpe broth
OD	Optical density
PBS	Phosphate-buffered saline
rpm	Revolution Per Minute
SD	Standard deviation
Temp.	Temperature
wk	Week
°C	Degree Celsius
%	Percentage
µg	Microgram

CHAPTER 1
INTRODUCTION

1.1 Rationale and Background

One of the critical issues in public health is gastrointestinal disorders, as foodborne diseases are common (WHO, 2015). Furthermore, these diseases are the cause of several gastrointestinal problems, illness and death in many countries (Bintsis, 2017). A bacterial infection is the most frequent foodborne illness that requires the use of antibiotics or other chemical-based drugs for treatment. These may cause side effects and destroy the balance of the microbiota in the gastrointestinal system. With regard to reports on foodborne illness, most studies have been associated with bacterial contamination, especially *Salmonella typhi*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Bacillus cereus*, including other strains; such as *Salmonella enteritidis* and *Vibrio parahaemolyticus* (Division of innovation and research (DIR), 2019). These have also been identified as the causative agents of foodborne diseases.

At present, products from nature, such as functional food or other related items have received much interest for improving health. Probiotics are one of the choices for health benefits, as they are beneficial bacteria that provide medicinal properties and are safe and contain fewer side effects. The Food and Agriculture Organization (FAO) of the United Nations and the World Health Organization (WHO) have defined as probiotic organisms are live microorganisms that when administered in adequate amounts they confer a health benefit on the host (Bielecka, 2006). These benefits include providing antimicrobial activity, acting as an anti-inflammatory agent and antioxidant, and alleviating lactose intolerance. Lactic acid bacteria (LAB) are a type of probiotic, which have been the most widely used in food products. LAB are "generally regarded as safe (GRAS)," as they play an essential role in the food fermentation process.

Moreover, LAB can produce antimicrobial substances, such as bacteriocins, lactic acid, acetic acid, and hydrogen peroxide (H_2O_2) (Hastings and Stiles, 1991). LAB sources are diverse, such as the natural environment, intestinal tracts of mammals, and fermented foods. Several research studies have isolated and identified LAB from foods; such as sausages (Ferrando et al., 2015), Harbin dry sausages (Han et al., 2017),

fermented meat products, traditional fermented fish products (Kopermsub and Yunchalard, 2010), fermented vegetables (Yasanga, 2008), and other food products. In Thailand, including fermented sausage (Mhom) (Samappito, Leenanon and Levin, 2011), fermented meat (Nham), fermented fish (Pla Som) (Sanpa, Sanpa and Suttajit, 2019), fermented food, and vegetable and fruit products (Yasanga, 2008). The screening and isolation of microorganisms from natural sources have always been the most powerful means for obtaining valuable strains for health products. Furthermore, the crucial factors for considering an appropriate probiotic are its survival through the digestive system, attachment to the intestinal epithelium, maintaining good viability, capable of exerting a beneficial effect, and safety. Many researchers have examined the survivability of probiotics, and some LAB strains are unable to survive through gastrointestinal conditions. However, some studies reported that prebiotics could improve the growth of probiotics (Wada et al., 2005). Researchers tried to isolate and characterize new probiotic strains from various natural sources, including fermented foods. Only a few reports that established the test to show the strong inhibitory activity when probiotics and pathogens were co-incubated together.

Prebiotics are non-digestible food ingredients that allow beneficial changes. They may help the survival rate of probiotic strains, enhance the host's defense mechanisms, increase resistance to various health disorders, modify problematic issues of the human gastrointestinal tract (Mountzouris et al., 2006), and improve the host's health (Gibson and Roberfroid, 1995). Inulin is prebiotic, which is a natural storage fructan carbohydrate that can be used for nutritional purposes; such as low-calorie soluble dietary fiber and for stimulating the growth of probiotic gut bacteria, as well as for mediating sugar and lipid metabolism (Wada et al., 2005; Judprasong et al., 2011). The study investigated the use of inulin powder to be developed as a synbiotic product, a combination of selected LAB with inulin powder extracted from Jerusalem artichoke (JA) (*Helianthus tuberosus*). JA is a plant species used in the commercial production of prebiotics, which can provide the greater stability of probiotics, so it can be considered as a potential source for developing healthy food (Mansouri et al., 2016). Previous research reported that JA has high inulin in the tuber, demonstrating the beneficial effects of health, such as the antimicrobial activity and antioxidant capacity (Dias et al., 2016). Synbiotics are a combination of probiotics and prebiotics, which can increase the

effectiveness of probiotics for positive health benefits. Therefore, this research focused on the isolation and characterization of LAB from local Thai fermented foods and screening isolated LAB for probiotic properties. Characterization of the selected potential probiotic strains was also performed. The freeze-dried synbiotic powder was developed by combining the selected probiotic strain and inulin extracted from JA. The stability of the prepared synbiotic powder was also investigated for further development as a synbiotic product exhibiting an anti-foodborne bacterial property.

1.2 Objectives of this study

The overall aim of this research was to develop a synbiotic product from local isolated probiotic strains for anti-foodborne pathogens. The specific objectives were:

1.2.1 To isolate and characterize LAB from local Thai fermented foods.

1.2.2 To screen LAB for probiotic properties.

1.2.3 To characterize selected potential probiotic strains.

1.2.4 To develop a synbiotic powder by using a freeze-drying method.

1.3 Anticipated outcomes

1.3.1 Obtaining novel strains of LAB for anti-foodborne pathogens that could be used as probiotic starters for health products.

1.3.2 Obtaining synbiotic powder containing an antimicrobial activity for anti-foodborne pathogens.

1.4 Scope of the study

The scope of this research consisted of the isolation and characterization of the LAB from local Thai fermented foods. Eighty-three isolates of the obtained LAB were then screened for their probiotic properties comprising anti-foodborne pathogens, acid, and bile salt tolerances. Then, the selected promising LAB isolates were evaluated for the potential probiotic strains, followed by the test of antimicrobial activity of the cell-free supernatant after neutralization.

The safety consideration, the selected LAB isolates were then tested for antibiotic susceptibility, hemolytic activity, and DNase activity. Then, the biochemical characterization and identification of the selected LAB with API 50 CHL and the 16S rDNA gene

sequences were determined. In addition, the selected LAB isolates were tested for Caco-2 cell adhesion and antimicrobial activity with a co-culture assay of probiotic strains and some pathogenic bacteria. In the step of the development of the synbiotic powder, the selected probiotic strain and inulin powder extracted from JA were prepared by the freeze-drying method. Probiotic viability under the simulated gastrointestinal conditions of the obtained freeze-dried synbiotic powder was investigated. Finally, the stability of the prepared synbiotic powder during the storage and anti-foodborne bacterial activity was evaluated. The overall of this research work was shown as the diagram in Figure 1.1.

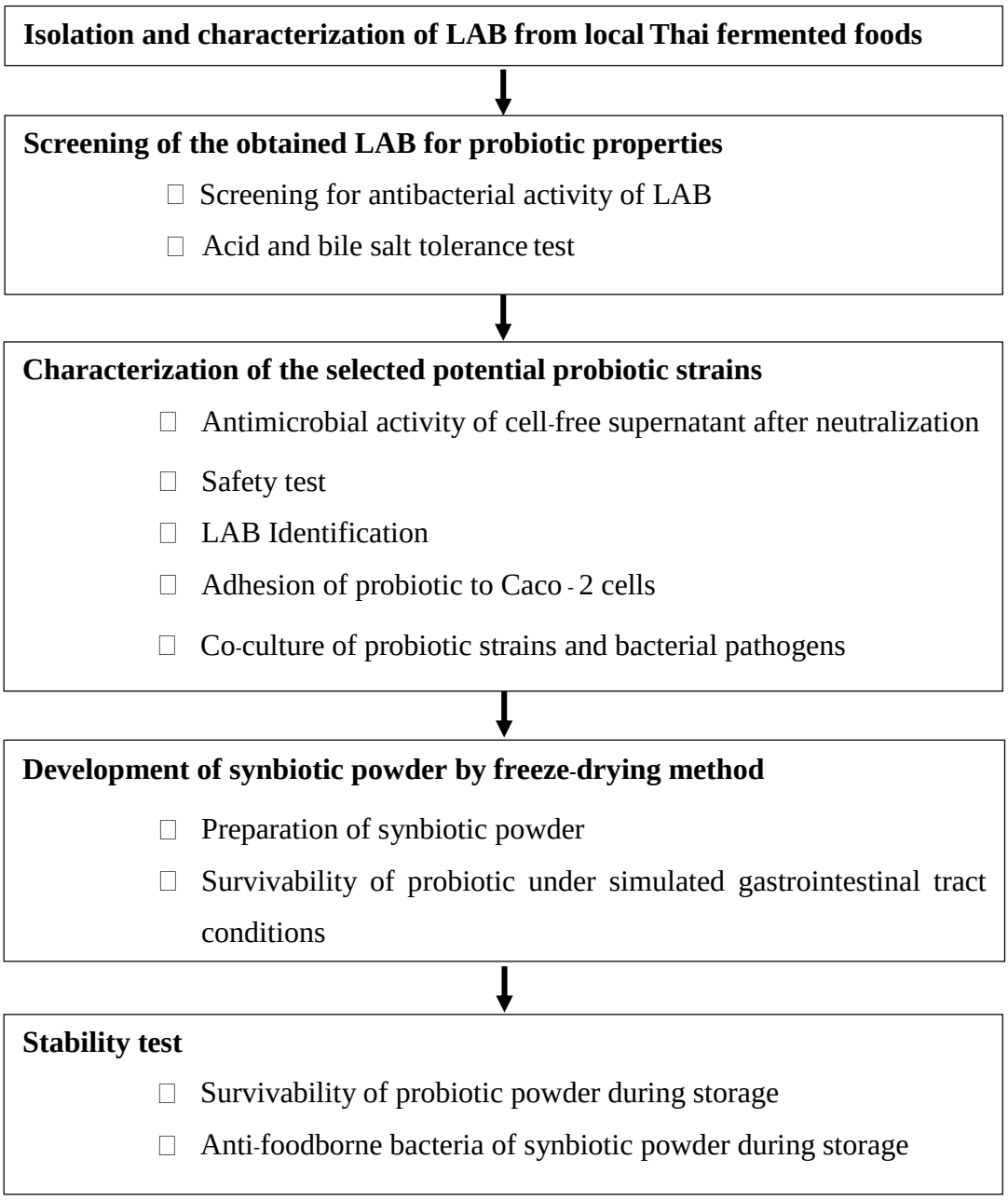


Figure 1.1 Schematic diagram of this research

CHAPTER 2
LITERATURE REVIEWS

2.1 Probiotics

2.1.1 Definitions of probiotic

The term probiotic was initially derived by the combination of a Latin preposition pro (meaning "for" or "in support") with Greek noun bios (meaning "biotic" or "life"). However, ever since the first proposed definition of probiotics as substances secreted by one microorganism stimulates another microorganism (Lilly and Stillwell, 1965).

Watson and co-worker define that probiotics are commonly defined as a viable microorganism that exhibits a beneficial effect on the host's health when they are ingested. However, the health benefits are strain-specific and not species- or genus-specific. Many health effects attributed to probiotic microorganisms are related, among others, to the Gastrointestinal tract (GIT), showing the ability to survive through the upper GIT, and be capable of surviving and growing in the intestine. Also, probiotics are safe, produce antimicrobial substances, and adhere to human intestinal cell lines and colonize the intestine (Watson and Preedy, 2015).

Probiotics are non-pathogenic microorganisms, on which the ingested inadequate amount exerts a positive health benefit on the host (Bielecka, 2006).

Probiotics are defined as the live microorganisms which are a dietary supplement that possesses several effects on the host by their activities (Guarner and Schaafsma, 1998). They can survive in the bile acids condition and form colonies in the gastrointestinal tract without harming the host. Although many microorganisms exist in the human gut, only a few strains of microorganisms have a probiotic characteristic. They are mainly *Lactobacillus* and *Bifidobacterium.*

Food and Agriculture Organization (FAO) and the World Health Organization (WHO) defined that probiotics are described as live microorganisms that are most effective on the health of humans when consumed in adequate amounts (Fao et al., 2002). Metabolically active bacteria are useful in the human gut only if the number of microorganisms per gram of food is over 10^6 at the time of consumption (Gilliland, 1989).

2.1.2 The criteria to be considered as a probiotic

The criteria to be considered as an excellent probiotic bacteria are as follows as essential points. The probiotics are generally considered safe to the host and the environment (Watson and Preedy, 2015).

It should be a strain that can benefit the host animal, for instance, increased growth or disease resistance.

It should be non-pathogenic, nonallergic, nontoxic, and noncarcinogenic.

It should be present in a viable form and, if possible, in large numbers.

It should survive and metabolize in the gut environment- for example, and it should be resistant to low pH, bile salts, organic acid.

It should be stable and able to remain viable for longer periods under storage and field conditions.

The most common probiotics are *Lactobacillus* species and *Bifidobacterium* species, but microorganisms, including *Escherichia coli, Streptococcus, Enterococcus,* and *Saccharomyces* species, have some characteristics of probiotics that have shown in Table 2.1 (Petre, 2012).

Table 2.1 Common probiotics

***Lactobacillus* species**	**Other lactic acid bacteria**	**Non-lactic acid bacteria**	***Bifidobacterium* species**
L. acidophilus	*Enterococcus faecium*	*Bacillus subtilis*	*B. adolescentis*
L. bulgaricus	*Streptococcus thermophilus*	*Escherichia coli strain nisslle*	*B. bifidum*
L. casei		*Saccharomyces boulardii*	*B. breve*
L. rhamnosus GG		*Saccharomyces cerevisiae*	*B. infantis*
L. plantarum			*B. longum*
			B. thermophilus

Source: Smith and Jones (2012)

2.1.3 Guidelines for probiotics

In order to claim that a food has a probiotic effect, it should be as follows: the first is the evidence to suggest that probiotic effects for human health are strain-specific. Specification of the bacteria must be established using the most current and valid methodology. The second is that *in vitro* tests are critical to evaluate the safety of probiotic bacteria. Moreover, *in vitro* tests are helpful to gain knowledge of strains and the mechanism of the probiotic effect. Appropriate target-specific *in vitro* tests that correlate with *in vivo* studies are recommended. For example, *in vitro* bile salts resistance was related to gastric survival *in vivo* (Conway, Gorbach and Goldin, 1987). Finally, the requirements for proof that a probiotic strain is safe and without contamination in its delivery form. (Adams and Marteau, 1995). The use of probiotics in food or supplement products for safety associated with side effects (Marteau, 2001)

are systemic infections, harmful metabolic activities, excessive immune stimulation in susceptible individuals, and gene transfer (Marteau, 2001).

In recognition of the importance of assuring safety, even among a group of bacteria that are Generally Recognized as Safe (GRAS), they recommend that probiotic strains be characterized at a minimum with the following tests:

2.1.3.1 Determination of antibiotic resistance patterns

2.1.3.2 Assessment of specific metabolic activities

2.1.3.3 Assessment of side-effects during human studies

2.1.3.4 Epidemiological surveillance of adverse incidents in consumers

2.1.3.5 Suppose the strain under evaluation belongs to a species that is a known mammalian toxin producer. In that case, it must be tested for toxin production by the EU Scientific Committee on Animal Nutrition (SCAN, 2000).

2.1.3.6 If the strain under evaluation belongs to a species with known hemolytic potential, the determination of haemolytic activity is required. Moreover, evaluation of the lack of infectivity by a probiotic strain in immunocompromized animals would add a measure of confidence in the safety of the probiotic. In some cases, animal models exist to substantiate *in vitro* effects and determine probiotic mechanisms and use these before human trials (Fao et al., 2002).

2.1.4 Mechanisms of actions

Probiotics have been demonstrated to have multiple beneficial effects for human health, principally in the preventing infectious diseases and food allergies, reducing serum cholesterol, promoting anti-carcinogenic activity, and enhancing systemic and mucosal immunity (Figure 2.1). In general, log 6 - log 7 of probiotic bacteria per ml or g of food has been recommended for an exhibition of health benefits (Lähteinen et al., 2010).

At the gut level, some of the benefits exerted by probiotics are maintaining a beneficial balance of the intestinal microbiota, reinforcing the intestinal barrier, antimicrobial effect against enteric pathogens and modulation of the mucosal immune system.

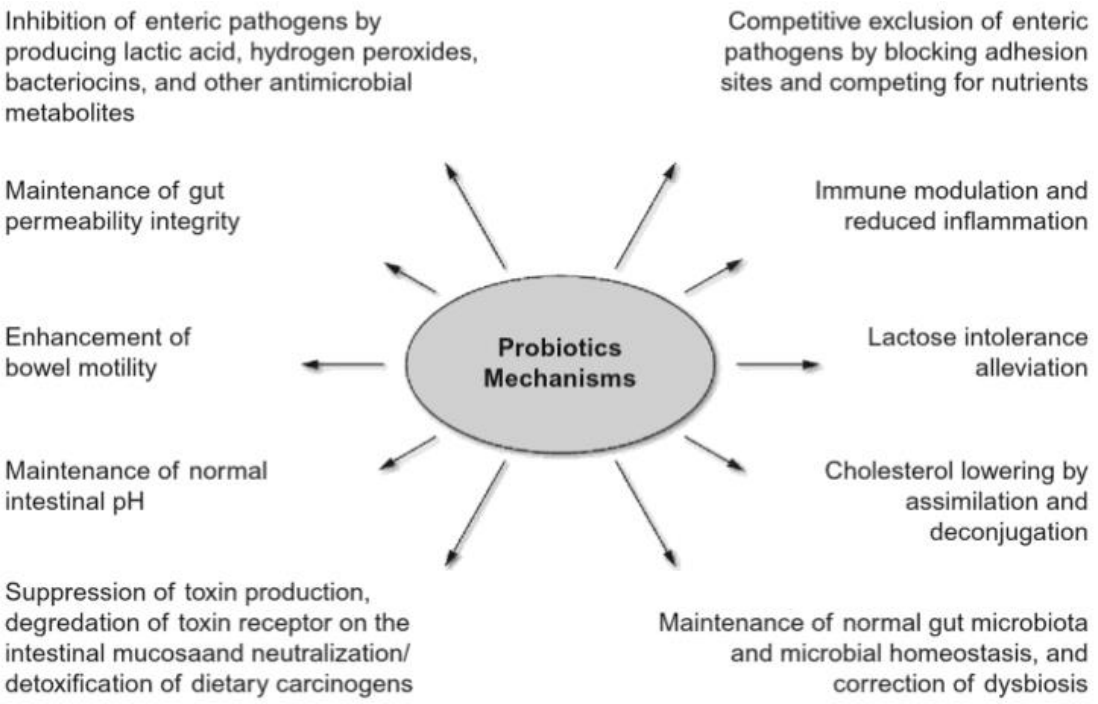


Figure 2.1 Proposed mechanisms of actions underlying the health effects of probiotics

Source: Watson and Preedy (2015)

Probiotic microorganisms may exert their protective effect against intestinal pathogens through various immune and non-immune mechanisms.

2.1.4.1 Non- immune mechanisms induced by probiotics to improve the gastrointestinal barrier for the previous research reported that probiotics could shape the intestinal microbiota, favoring the development of beneficial bacteria and limiting the growth of undesired microorganisms. They achieve this effect and protect the host through different mechanisms, such as the secretion of antibacterial substances, their metabolic activity, and competition with pathogens for adhesion sites in the intestinal epithelium or the nutrients necessary for pathogens survival (de Moreno de LeBlanc et al., 2008). The second is that probiotics can produce antimicrobial compounds and induce the synthesis and secretion of antimicrobial peptides by enterocytes and paneth cells. These compounds include organic acids of low molecular weight (< 1,000 Da) and with a broad spectrum of action and antimicrobial proteins (bacteriocins) with a more restricted spectrum. Lactic and acetic acids are organic acids generated as a result

of fermentative metabolism of hexoses and are considered primarily responsible for the inhibitory effect of different probiotic strains against gastrointestinal pathogens such as *Helicobacter pylori*, *E. coli* O157: H7, *S. flexneri*, *Clostridium difficile*, *S. enterica* serovar *Typhimurium* and *S. Typhi* (Servin, 2004; Kaushik et al., 2009).

Moreover, many lactobacilli and bifidobacteria are reported to secrete antimicrobial peptides called bacteriocins. These are usually active against closely related bacteria that are likely to reside in the same ecological niche. However, numerous in vitro studies indicate that certain probiotic strains isolated from the feces of adults and children can synthesize broad-spectrum antimicrobial proteins, active against Gram positive and Gram negative bacteria and yeasts. These include critical gastrointestinal pathogens such as *C. difficile*, *Listeria monocytogenes*, *Staphylococcus aureus*, *E. coli*, *Salmonella*, *Shigella*, *Vibrio cholerae*, *H. pylori*, *Campylobacter coli* and *Arcobacter butzleri* (Collado et al., 2005a; Collado, Hernandez and Sanz, 2005; Cleusix et al., 2007; Spinler et al., 2008). Probiotic bacteria and commensal microbiota are also capable of stimulating the production of defensins and other antimicrobial peptides, by enterocytes and Paneth cells. However, some pathogens are developing resistance mechanisms to this barrier by reducing these peptides' expression or evading the enzymatic attack (Salzman et al., 2003). The thirds, probiotics stimulate mucus secretion for maintaining intestinal barrier homeostasis with the microbiota, minimize contact between luminal microorganisms and the intestinal epithelial cell surface. This is accomplished through the production of mucus, antimicrobial proteins, and S-IgA. Goblet cells are specialized epithelial cells that secrete mucin glycoproteins. These mucins form a mucus layer that extends up to 150 μm from the intestinal epithelial cell surface (Hooper and Macpherson, 2010). The last mechanism, competition for adhesion sites form the several in vitro models based on cellular lines (Caco-2, HT29-MTX, T84, INT407) and intestinal mucus, have been used to demonstrate the ability of different probiotic strains to inhibit the adhesion, invasion, or to displace pathogens previously adhered to the cell layer, such as *Salmonella enterica* serovar *Typhimurium*, *Escherichia coli*, *Enterobacter sakazakii*, *Clostridium difficile* (Bernet et al., 1993; Matsumoto et al., 2002; Collado et al., 2005b; Liu et al., 2010; Martins et al., 2010).

2.1.4.2 Probiotics modulate the immune system, preventing or attenuating diverse enteric infectious diseases. The intestine is one of the gateways to the body, the

reason why it is particularly vulnerable to infection. In the intestinal mucosa, there are continuous antigenic challenges from birth to death, food antigens, antigens from the abundant normal microbiota, and pathogens. The integrity layer in healthy individuals is maintained even though the entire surface is removed and renewed every 2 - 3 days. This is possible because the gastrointestinal tract possesses a defense system that prevents infections from occurring microorganisms and maintains an efficient exchange between the body and nutrients. This system consists of physical, chemical, mechanical, and immunological barriers. The immunological barrier is constituted by a complex and coordinated network of epithelial and immune cells, interacting with each other via cellular messengers and by antibodies, especially those of Ig A class. Probiotics and other intestinal bacteria may interact with the mucosal-associated immune system by inducing pro-inflammatory and anti-inflammatory cellular and humoral responses. The effects produced by these bacteria may vary greatly, depending on the species, strain, dose and, matrix in which the probiotic is administered. Therefore, the researcher must conduct an individualized and comprehensive assessment of each probiotic strain under study. The study demonstrated that oral administration of fermented milk with *Lactobacillus casei, Lactobacillus acidophilus*, or a mixture of both microorganisms to Swiss albino mice, enhanced systemic immunity in the host enhancing phagocytic and lymphocytic activity (Perdigon et al., 1988). The promising findings reported the probiotic suspension of *L. casei* protection against *Salmonella* and *E. coli* infections in mice. They found that *L. casei* induced an increase of specific antibodies against both pathogens after two days of feeding and increased cellularity in lamina propria of the small intestine after seven days of probiotic administration. These increases were accompanied by a slight inflammatory response only seven days post treatment. A significant result was the demonstration that IgA increases were firmly related to the dose and that the most pleasing effect was obtained with a dose of $2{,}4 \times 10^9$ cells (Perdigon, Alvarez and Pesce de Ruiz Holgado, 1991).

Perdigón et al. reported that the immunostimulatory properties of both natural and heated standard yoghurts and demonstrated that natural yoghurt was also able to increase specific and nonspecific defense mechanisms against *Salmonella*, enhancing resistance to *Salmonella Typhimurium* infection. The data show that a specific probiotic product’s protective capacity against pathogens depends on the type

of infectious agent and mainly on the infective dose since yoghurt is a very useful activator of the nonspecific host defense mechanisms. Other studies found a similar effect against *Salmonella* and *E. coli* infections using other probiotic strains and different animal models (Shu et al., 2013; Gill et al., 2001; Mountzouris et al., 2009).

2.1.5 Sources of probiotic

Many microorganisms involved in food fermentation have been reported as probiotics, such as *Lactobacillus, Bifidobacterium, Pediococcus, Lactococcus, Propionibacterium, Bacillus, Enterococcus*, and some yeasts (Missaoui et al., 2019). Various research in the probiotic area has achieved significant progress in selection and characterization of specific probiotic cultures and confirmed the health benefits associated with them. Traditionally, fermented foods are the primary source of probiotics and hence one of the major dietary supplements.

Products containing probiotic bacteria generally include foods and supplements. Fermented milk products are the most traditional source of probiotic strains of lactobacilli. However, commercial probiotic lactobacilli have also been added to meat products, snacks, fruit juice and many probiotics such as yogurt, kefir, milk soy, almond milk, juice, kimchi, and cereal bar. Probiotic properties of *Lactobacillus plantarum* isolated from kimchi has been reported and is also found to prevent the growth of *Helicobacter pylori*. Probiotic strain *Pediococcus pentosaceus* CIAL-86 isolated from wine shows anti-adhesion activity against *Escherichia coli* CIAL-153, indicating its probiotic potential. *L. acidophilus* La-5 produces conjugated linoleic acid (CLA), an anti-carcinogenic agent.

2.2 Lactic acid bacteria

Lactic acid bacteria (LAB), as one strain of probiotics. They consist of several bacterial genera within the phylum Firmicutes. The genera *Lactobacillus, Lactococcus, Lactosphaera, Streptococcus, Pediococcus, Enterococcus, Carnobacterium, Melissococcus, Leuconostoc, Tetragenococcus, Oenococcus, Vagococcus,* and *Weissella* are recognized as LAB. There are mainly gram-positive, anaerobic, non-sporulating, and acid and bile tolerant bacteria that can ferment nutrients primarily into lactic acid. They are found in various environmental habitat, including dairy, meat, vegetable, cereal, and plant. They are also found naturally in parts of human and animal gastrointestinal tracts

(*Lactobacillus acidophilus, L. gasseri, L. johnsonii, L. plantarum, Streptococcus agalactiae, Enterococcus faecalis*), oral cavities (*S. mutans, Bifidobacterium longum*), and vaginal cavities (*B. longum, S. agalactiae, L. crispatus*). LAB are one of the most industrially relevant groups of bacteria. These organisms could be applied in food industries, health improvements, and macromolecules, enzymes, and metabolites (Ekundayo, 2014).

LAB are present in most of the fermented foods and starter culture of LAB that are industrially important in food safety (Sathe and Mandal, 2016). These are desirable microflora of the GIT and are thus 'generally regarded as safe' (Tannock, 1997). They are involved in fermentation and the dominant microflora of fermented products. They play an essential role in inhibiting foodborne pathogens and spoilage microorganisms (Nur and Aslim, 2010). The antimicrobial effect of LAB is mainly due to their lactic and organic acid production, causing the pH of the growth environment to decrease. Low pH induces organic acids to become lipid soluble and diffuses through the cell membrane into the cytoplasm (Kuipers, Buist and Kok, 2001).

LAB has been cited to be part of the human microbiota. The neonates receive their microbiota primarily in labor and later from the environment (Edwards and Parrett, 2002). LAB dominates the microbiota of the full-term neonate, especially when breastfed with a health promoting effect on the child. The isolated LAB from human milk (Heikkila and Saris, 2003). Martín et al. (2003) detected *L. gasseri* from breast-feeding mothers and children in pairs and observed coccoid (Martín et al., 2003). LAB is regarded as a main group of probiotic bacteria. Several *bifidobacteria, lactococci*, and *lactobacilli* are held to be health-benefiting bacteria, but little is known about the probiotic mechanisms of gut microbiota. LAB constitutes an involved host metabolism and integral part of the healthy gastrointestinal microecology (Fernandes, Shahani and Amer, 1987).

Moreover, LAB and other gut microbiota ferment various substrates into short chain fatty acids and other organic acids and gases. LAB synthesizes enzymes, vitamins, antioxidants and, bacteriocins. With these properties, the intestinal LAB constitutes an essential mechanism for the metabolism and detoxification of foreign substances entering the body (Bandyopadhyay and Mandal, 2014). The study has been presented that some *lactobacilli* can stimulate the immune system on the gut mucosal surface via

localized GIT lymphoid cell foci. Many mechanisms work to prevent harmful bacteria from growing on and attaching to the intestinal epithelium with the production and secretion of antimicrobial agents (Reid, 2001), adherence via competition for the binding sites, and steric hindrance and barriers interfering with pathogens hence promoting the elimination of harmful bacteria. Some studies reported that vaginal LAB strains being able to self-aggregate in a process mediated by surface proteins or lipoproteins, depending on the strain (Zárate and Nader-Macias, 2006). The study for understanding the full effect of probiotics given the extreme complexity of the biological systems of humans and their interactivity. Studies conducted on bacteria's beneficial effect on human health cover only a segment of the human ecosystem (Shanahan, 2003).

LAB derived probiotics have potential health benefits in the following properties such as diarrheal diseases (Shornikova et al., 1997), inflammatory bowel disease (Harish and Thomas, 2006), prevention of colon cancer, inhibiting the growth of *H. pylori* (Hamilton-Miller, 2003), Lactose intolerance (Saltzman et al., 1999) and other disorders (Harish and Thomas, 2006). Moreover, LAB as a source of low-calorie sweeteners, low-calorie sugars produced from LAB has attracted the interest of consumers. They can be incorporated directly into foods or be produced in the food by LAB, leading to the production of foods containing (Patra, Tomar and Arora, 2009). LAB are very promising sources for products and applications, especially those that satisfy the increasing consumer demands for natural products and functional foods (Figure 2.2).

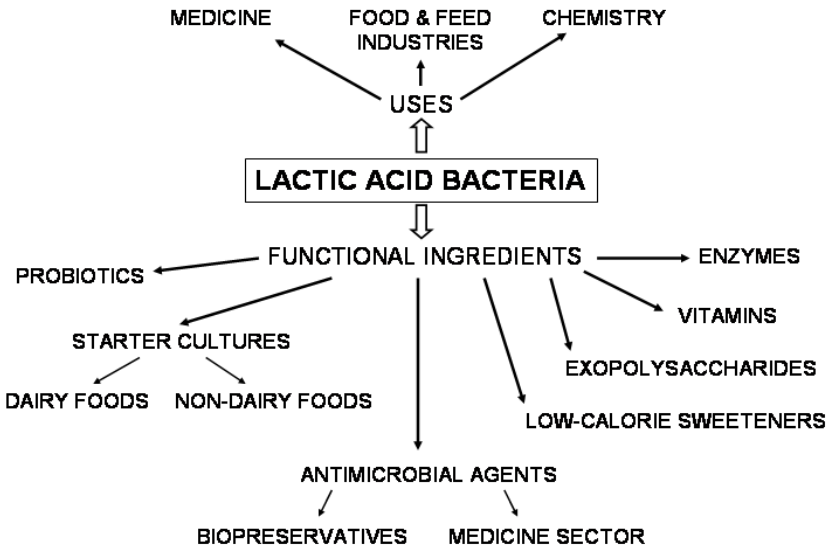


Figure 2.2 Uses and Functional Ingredients of Lactic acid bacteria

Source: Panagiota Florou - Paneri (2013)

2.3 Fermented foods

Fermentation is a food preservation technology known to humankind that is considered an essential determining factor to control microbial growth, improve the nutritional value of food and digestibility, and food safety (Tassou, Panagou and Nychas, 2010).

Most of the global fermented foods are known to be fermented by both functional and non-functional microorganisms (Franz et al., 2014). These microbes alter the biochemical constituents, thereby improving the flavor, aroma and digestibility while imparting nutritional and pharmacological values in some fermented foods. Although, some fermented foods are popular and promoted globally for their functional, nutraceutical, and therapeutic properties. Numerous reviews were published on the biological, chemical and nutritional components of fermented foods from various countries (Rhee et al., 2011; Dickey, 2005; Tamang, Watanabe and Holzapfel, 2016; Oguntoyinbo et al., 2011). Fermented foods have unique functional properties due to functional microorganisms, which possess probiotics properties, antioxidant, antimicrobial, peptide production. Health benefits of some global fermented foods are a synthesis of nutrients, prevention of cardiovascular disease, gastrointestinal disorders, diabetes, allergic reactions, prevention of cancer, among others. Fermentations

involving the production of lactic acid are generally safe. Lactic acid fermentations include those in which the fermentable sugars are converted to lactic acid by lactic acid organisms such as *Bifidobacterium bifidus, Lactobacillus plantarum, Lactobacillus brevis, Lactobacillus acidophilus, Lactobacillus bulgaricus,*

Leuconostoc mesenteroides, Streptococcus thermophilus, Pediocccus cerevisiae, Streptococcus lactis, Lactobacillus citrovorum. (Steinkraus, 1994).

LAB mostly species of *Lactobacillus, Enterococcus, Lactococcus, Leuconostoc, Pediococcus, Weissella* are widely present in many fermented foods (Axelsson et al., 2012). Species of *Bifidobacterium, Brevibacterium, Brachybacterium,* and *Propionibacterium* are isolated from cheese, and species of *Arthrobacter* and *Hafnia* from fermented meat products (Bourdichon et al., 2012). Species of *Bacillus* are also present in legume-based fermented foods (Kubo et al., 2011). Some LAB as starter culture in fermented foods such as *Lb. plantarum* from Kivunde (Kimaryo et al., 2000), *Pediococcus pentosaceus* from pork sausages (Kingcha et al., 2012), *Lb. plantarum* and *Lb. reuteri* from fermented fish (Plaa-som), *Weissella cibaria*, and *W. koreensis* from Kimchi, *Lb. delbrueckii* subsp. Bulgaricus and *Streptococcus thermophiles* from yogurt (Michaylova et al., 2007) and various types of foods. Fermented foods generally have a safety record in the developing world where people manufacture the foods without training in microbiology or chemistry in unhygienic, contaminated environments. Some fermented foods and beverages have health benefits due to the presence of functional microorganisms. Although, some fermented foods and beverages are marketed globally as health foods, therapeutic foods, nutraceutical foods, functional foods, bio-foods. However, reliance on fewer providers of fermented foods is also leading to a decline in the biodiversity of microorganisms. Therefore, animal models' validation of health claims and clinical trials of some common fermented foods may be studied in details (Tamang et al., 2016).

2.4 Foodborne pathogens

Foodborne pathogens are causing many diseases with essential effects on human health and the economy. Foodborne illnesses are diseases caused by the ingestion of food. The global incidence of foodborne disease has been reported that people died from diarrhoeal diseases, and a high proportion of these cases can be attributed to

contamination of drinking water and food (WHO, 2002). Some foodborne diseases are well recognized but are considered. They were emerging because they have recently become more common. Though various foodborne pathogens have been identified for food-borne illness, *Salmonella*, *Campylobacter*, *Listeria monocytogenes*, and *Escherichia coli* O157: H7 have been generally found to be responsible for the majority of food-borne outbreaks (Chemburu, Wilkins and Abdel-Hamid, 2005).

Food-borne illnesses and diseases are transmitted to humans from infectious organisms in food and water, generally resulting in gastrointestinal symptoms that vary in severity and duration. Bacteria, viruses, or parasites may cause enteric diseases. (Lines M.P.H., 2020). Bacteria are the most common cause of foodborne diseases and exist in various types, shapes, and properties. Some pathogenic bacteria are capable of spore formation and thus, highly heat-resistant toxins *(*e.g., *Staphylococcus aureus, Clostridium botulinum*). Most pathogens are mesophilic, with an optimal growth temperature range from 20 °C to 45 °C.

Food-borne bacteria that cause illness in humans are diverse in their physiology, their pathogenesis, and the severity of the illness caused. In the digestive system, they can be subjected to competition and specific inhibitory mechanisms by probiotic bacteria that reduce their ability to cause disease. Also, probiotic bacteria can help to reduce the number of pathogenic bacteria being consumed. (Potter and Morris, 2013).

Food poisoning is considered one of the most common causes of illness and death in developing countries. The report is associated with bacterial contamination, especially members of Gram negative bacteria like *Escherichia coli, Salmonella typhi,* and *Pseudomonas aeruginosa*. Other Gram positive bacteria, including *Staphylococcus aureus* and *Bacillus cereus*, have also been identified as the causal agents of food-borne diseases. Therefore, this study examined the anti-bacteria activity of selected probiotic bacteria against food-borne pathogenic bacteria. Six pathogenic bacteria were used in this study.

2.4.1 *Escherichia coli*

Escherichia coli (*E. coli*) (Griffin and Tauxe, 1991; Lynch, Tauxe and Hedberg, 2009) are a gram-negative bacteria that member the *Enterobacteriaceae* family, its ability to adapt and colonize a diverse array of reservoirs, including open-air environments and the GIT of mammals. Such flexibility is permitted through facultative

respiration, during which citrate, NO_2, and NO_3 take oxygen in the electron transport chain. Moreover, *E. coli* bacteria are mesophilic organisms that replicate at temperatures of 7 to 45 °C under optimal temperature conditions. The pathogen can replicate at pH values of 4 to 10 and in the presence of up to 8% sodium chloride (Fox et al., 2004).

E. coli bacteria usually live in the intestines of humans and animals; they can be found in water, foods, and the environment. Most *E. coli* are harmless and are an essential part of a healthy human intestinal tract. However, some *E. coli* can cause illness, diarrhea, or illness outside of the intestinal tract. The types of *E. coli* that can cause diarrhea can be transmitted through contaminated water or food, or contact with animals or persons (Lynch et al., 2009), and other illnesses (Griffin and Tauxe, 1991).

2.4.2 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa (*P. aeruginasa*) is a Gram-negative, rod-shaped, monoflagellated bacterium and a sporogenous. It has a pearlescent appearance and a grape-like odour. *P. aeruginosa* is a ubiquitous microorganism that can survive under various conditions. *P. aeruginosa* grows well at 25 °C to 37 °C, and its ability to grow at 42 °C helps distinguish it from many other *Pseudomonas* species. *Pseudomonas* is a type of bacteria that is commonly found in the environment. The many different types of *Pseudomonas*, the one that most often causes infections in humans, is called *Pseudomonas aeruginosa*, which can cause infections in the lungs, blood, or other parts of the body after surgery (Wu et al., 2015). *P. aeruginasa* can infect a range of tissues and sites and is one of the top six infectious disease threats that cause severe infections in immunocompromised patients or whose natural defenses are otherwise breached. The pathophysiology of infections is complex, as shown by the clinical diversity of diseases associated with this organism. These virulence factors facilitate tissue invasion and systemic spread and include endotoxins, exotoxins, pili and flagella, vascular permeability factors, and excreted enzymes. *P. aeruginosa* spoils a wide range of food stuffs such as meat, fish, milk and dairy products, water contamination, fruit, and vegetables (Singh, 2017).

2.4.3 *Salmonella enteritidis*

Salmonella is a Gram-negative, facultatively anaerobic bacteria belonging to the family of *Enterobacteriaceae*. *Salmonella* spp. is found in the environment, but the intestinal tract of animals is the main habitat of the bacteria. *Salmonella* contamination

occurs through the consumption of contaminated foods like milk, egg, and poultry meat. Twenty percent of world poultry products are contaminated with *Salmonella*, and they can persist for a long time in the animal and human environments and facilities through biofilm formation. In most salmonellosis outbreaks resulting from poultry products consumption, *Enteritidis* and *Typhimurium* serovars have been isolated (Afshari et al., 2018). *Salmonella enteritidis* bacterium symptoms usually have abdominal cramps, fever, and diarrhea after consuming a portion of contaminated food or beverage. The illness usually lasts 4 to 7 days, and most persons recover. The infants, elderly may have a more severe illness. In these patients, the infection may spread from the intestines to the bloodstream and then to other body sites and cause death (Singh, 2017).

2.4.4 *Salmonella typhimurium*

Salmonella typhimurium (*S. Typhimurium*) is a Gram-negative bacterium found in the intestinal. Its toxicity is due to an outer membrane consisting mainly of lipopolysaccharides which protect the bacteria from various condition. They can cause gastroenteritis in humans and other mammals. When the bacterial cells enter epithelial cells lining the intestine, they cause host cell ruffling, which temporarily damages the microvilli on the cell's surface. A causes a rush of white blood cells into the mucosa, which throws off the ratios between absorption and secretion, and leads to diarrhea (Everest et al., 1999). *Salmonella Typhimurium* invades the host through the Peyer's patches, aggregating of lymphoid tissue in the small intestine, and evokes an inflammatory response. However, when phagocytes take up the pathogen, it remains variable and is transported to systemic tissue. (Jørgensen, van Raaphorst and Veening, 2013).

Salmonella spp. have the ability to metabolize nutrients by the respiratory and fermentative pathways. *Salmonella* strains have an optimum pH for sustained growth between 6.5 and 7.5. In addition, the optimal temperature is between 35 and 40 °C. However, dependent on the strain and the type of food matrix. *S. enterica* serovar *Enteritidis*, *S. enterica* serovar *Typhimurium*, and *S. enterica* serovar *Newport* are the most frequently isolated *Salmonella* serovars from human salmonellosis cases (Singh, 2017).

2.4.5 *Bacillus cereus*

Bacillus cereus (*B. cereus*) *is* a gram-positive, aerobic spore-forming bacterium found in soil, vegetables, and processed foods. *B. cereus* food poisoning may occur when foods are prepared and held without adequate refrigeration for several hours before serving, with *B. cereus* reaching $>10^6$ cells/g (Stenfors Arnesen, Fagerlund and Granum, 2008). The ubiquitous nature of these organisms makes contamination of food materials a common occurrence. Arguably an essential characteristic of *Bacillus* spp. is their ability to form refractile endospores. Two types of illness of the consumption of foods contaminated with *B. cereus*, the first is the diarrheal syndrome, which has an incubation time of 4 to 16 h and is manifested as abdominal pain and diarrhea that usually subsides within 12 to 24 h and second is the of emetic syndrome, which has an incubation time of 1 to 5 h, causing nausea and vomiting that last for 6 to 24 h. The cause emetic *B. cereus* food poisoning, the food involved, will typically contain 10^5 to 10^8 cells/g. However, in some cases of *B. cereus* outbreaks, there appears to be a clear overlap of the diarrheal and emetic syndromes. As well as causing enteric illness, *B. cereus* has been responsible for postoperative infections. Some reports of neonatal infections due to *B. cereus* have been proposed that the systemic complications observed with these cases are associated with entero-toxins (Singh, 2017).

2.4.6 *Staphylococcus aureus*

Staphylococcus aureus (*S. aureus*) is Gram-positive bacteria that are cocci-shaped and grape-like morphology. *S. aureus* is a major bacterial human pathogen that causes various clinical manifestations (Lowy, 1998). *S. aureus* is found in the environment and is also found in normal human flora. *S. aureus* does not usually cause infection on healthy skin; however, if it is allowed to enter the bloodstream or internal tissues, these bacteria may cause various potentially dangerous infections (Lowy, 1998). *S. aureus*, the etiological agent of *staphylococcal* food poisoning, produces a cadre of extracellular pathogenic factors. *S. aureus* is one the most common bacterial infections, including bacteremia, skin and soft tissue infections, infective endocarditis, pulmonary infections, gastroenteritis, meningitis, toxic shock syndrome, and urinary tract infections. Depending on the strains involved and the site of infection, these bacteria can cause invasive infections and toxin-mediated diseases (Tong et al., 2015).

2.5 Methodology used in this probiotic study

2.5.1 Isolation and identification of candidate probiotic strains

Isolation and identification of candidate probiotics should have been done by preliminary screening. Strains of *Lactobacillus* have traditionally been used in the production of fermented dairy products and are generally recognized as safe (O'Sullivan et al., 1992). Furthermore, these bacteria are desirable members of the intestinal microflora (Berg, 1998). Table 2.2 shows a list of *Lactobacillus* strains isolated from foods that are usually considered probiotics.

Table 2.2 Some probiotic *Lactobacillus* strains isolated from foods

Probiotic strains	Sources	References
L. plantarum LP3	Kurdish cheese	Hashemi et al. (2014)
L. plantarum AF1	Kurdish cheese	Hashemi et al. (2014)
L. plantarum	Koozeh cheese	Hassanzadazar et al. (2012)
L. plantarum	White cheese	Mirzael and Barzgari (2012)
L. plantarum	Lighvan cheese	Mirzael et al. (2012)

Source: Watson and Preedy (2015)

2.5.2 Antimicrobial activity test

Antimicrobial activity is one of the criteria for screening probiotics. There is a various test for antimicrobial properties such as an agar spot test, agar well diffusion assay, co-culture assay. Antimicrobial substances produced by probiotic bacteria may have bacteriostatic or bactericidal effects on other microorganisms. These antimicrobial compounds include organic acids, fatty acids, hydrogen peroxide, bacteriocins, diacetyl, and acetoin. Numerous antimicrobial substances are produced by probiotic bacteria that have advantages in competition with harmful bacteria (Savage, 1992). Among organic acid produced by probiotic bacteria, lactic acid is the main metabolic end product of sugar fermentation. Furthermore, lowering the pH and through its undissociated form, lactic acid is also known to function as a permeabilizer of the Gram negative bacterial outer membrane.

Numerous studies were done to assess the antimicrobial activity of *Lactobacillus* strain. The in vitro production of inhibitory substances toward known pathogens by different probiotic strains has frequently been used to select candidate probiotics (Jafari, Rezaie and Alizadeh, 2011). LAB isolated and identified from kimchi can produce organic acids, and bacteriocins, all of which can have antimicrobial activity. *Lab. Curvatus, Lab. Pentosus, Leu. Mesenteroids, Leu. carnosum, Wei. cibaria, Wei. Kimchi, and Ped. pentosaceus* were isolated from kimchi. LAB showed antimicrobial activities against *E. coli, Bac. subtilis, Sal. enteritidis, Sal. typhi, Sal. paratyphi, Staph. aureus, Shigella boydii, and Shi. sonnei* depending on the strain (Ahn et al., 2003). The culture solution of *Streptococcus thermophiles* from kimchi inhibited *H. pylori*, which did not grow during the co-culture with these strains (Lee, et al., 2010). The study about cell-free culture supernatant of probiotic strains isolated from Kurdish cheese using the agar well diffusion method. All probiotics tested demonstrated inhibition against the enteric pathogens tested. However, *L. plantarum* LU5 showed the most effective inhibition of *Staphylococcus aureus*. The supernatant of *L. plantarum* LU5 was more effective against *E. coli* O157 H7 than *L. plantarum* LS5. *L. plantarum* strains and *L. paraplantarum* also demonstrated inhibition against *P. aeruginosa* and *Bacillus cereus*. Besides, in disk diffusion assay, all *Lactobacillus* strains had an inhibitory effect on potentially pathogenic microorganisms such as *S. aureus*, *E. coli*, *B. cereus*, and *P. aeruginosa*. In both agar well diffusion and disk assay, the diameter of inhibition zones of *Lactobacillus* strains was found to have disappeared when sodium hydroxide (neutralized to pH 6.5) was added to the cell-free culture supernatant. However, treated cell-free culture supernatant (treated with proteinase k and heat) of all *Lactobacillus* strains showed antibacterial activity against pathogens. Isolated *Lactobacillus* strains showed that all isolates were inhibitory against pathogens in pH 4 and that may be because of low pH and acidic conditions of culture. However, in pH 6.5, some were inhibitory against some pathogenic microorganisms (Jafari, Rezaie and Alizadeh, 2011).

Sugar fermentation in this method, followed by a reduction in pH due to lactic and other organic acids, is an important factor for the inhibition growth of pathogenic microorganisms. The antagonistic activity exhibited by various *Lactobacillus* strains was further evaluated by the well diffusion method. All isolates did not inhibit pathogenic bacteria growth when the neutralized cell-free culture

supernatants were applied directly to the agar diffusion method, except *L. farieminis*. On the other hand, non-neutralized cell-free culture supernatants of isolates inhibited the growth of some indicator bacteria.

In this research, to determine whether the inhibitory effects were due to acid or any other substances, a part of the cell-free culture supernatant was treated and used. The treated samples did not produce any zone of inhibition, indicating that the inhibitory substance was a single or a group of organic acids and acid derivatives and a bacteriocin that is not active at pH 6.5. Lactic acid bacteria are found to produce large quantities of organic acids. These organic acids in undissociated form diffuse through the pathogenic cell membrane. Then, diffused acids dissociate inside the cell to a degree depending on the intracellular pH. The proton ions released during the dissociation are reported to acidify the cytoplasm resulting in bacteriostasis and eventual death of the pathogenic bacteria. Additionally, the anionic part of the acid cannot diffuse freely through the cell wall and consequently accumulates inside the bacterial cell (Savage, 1992).

2.5.3 Acid and bile resistance

The potential probiotics for human health had acid and bile resistance to gastrointestinal conditions to promote health. The acid condition is the most negative effect on the viability of *Lactobacillus* strains during their passage through the human gastrointestinal tract condition. The probiotic strain that survives in the gastrointestinal tract must have acid tolerance. In conventional acid tolerance assay, the viability of probiotics is determined by exposing them to low pH for a while, during which the number of surviving organisms remaining is determined. The pH level in the stomach ranges from 1.5 to 4.5 depending on the intervals time of the digestive system, which can take up to 3 h. *Lactobacillus paraplantarum* and *Lactobacillus plantarum* strains isolated from cheese have been reported to the resistance under simulated gastric acid conditions. The residual cells were more than 50% of the initial cells of incubation at pH 2 or 3 after 2 h. *Lactobacillus paracasei* and *Lactobacillus casei* strains isolated from cheese displayed considerably higher survival at 120 min of exposure at pH 2.0 than their corresponding strains of origin.

In vitro study showed that *L. plantarum* strains isolated from Lighvan cheese were able to survive after 2 h of exposure at pH 1.55, where the number of final surviving bacteria ranges between 6.3 and 7.86 Log CFU/g. Moreover, *Lactobacillus*

fermentum and *L. plantarum* isolated from traditional white cheese were able to survive at pH 3 for 3 h. The study about *L. plantarum* and *Lactobacillus jensenii* were found to be more resistant to stomach conditions like low pH. *Lactobacillus* strains from Koozeh cheese were exposed to pH 2.0 and 3.0 conditions of the stomach for 3 h. In this study, the bacterial growth decreased with increasing duration at pH 2 and remained constant at higher pH.

Ingestion of probiotic strains with food raises the pH in the stomach to 3.0 or higher (Iñiguez-Palomares, Pérez-Morales and Acedo-Félix, 2008). Thus, raising the pH improved the survival of probiotic this could be a viable strategy for improving the survival of probiotics through the gastrointestinal condition.

Additionally, in the digestive system bile plays an essential role in lipid digestion; it emulsifies and solubilizes lipids and functions as the biological detergent (Hofmann and Roda, 1984). Bile tolerance of probiotics can be examined by incubating them for 24 h in a medium containing of bile salts and monitoring cell viability and pH before and after incubation (Goktepe, Juneja and Ahmedna, 2005). This assay was used by several authors to assess the bile resistance of native probiotic *lactobacilli.* All these studies reported a growth delay of *Lactobacillus* strains in the presence of bile salts that were strain specific. Bile tolerance of *Lactobacillus* strains isolated from Kurdish cheese was evaluated. These strains were resistant to 0.3% to 1% of bile salt after 24 h incubation. While all bacteria could tolerate 0.3% to 1 % bile salt, *L. plantarum* LP3 was the most resistant strain of all.

Moreover, *L. plantarum* isolated from Lighvan cheese were examined for their bile tolerance (0.3%) showed bile tolerant, with a final surviving population of ~9.0 log CFU/mL. Bile tolerance dependent on bile type and strain, with resistance levels ranging from bile concentrations of 0.125% to 2.0%.

2.5.4 Epithelial cell adhesion assay

The capability to adhere can provide information about the possibility of probiotic to colonize and may modulate the host immune system. Different mechanisms were reported about the adhesion of probiotics to intestinal epithelial cells. The in vitro ability to adhere to the intestinal epithelium is considered the critical parameter influencing their colonization (Savage, 1992). The study of *Lactobacillus* strains illustrated they possessed the capability to adhere to the intestinal epithelium cell.

Isolated lactobacilli strains expressed a higher in vitro adherence to Caco-2 cell line than the commercial *Lactobacillus* strains, and *L. plantarum* LS5 strain exhibited the highest adhesion (17.3%). This property could indicate a benefit and importance for bacterial maintenance in the human gastrointestinal tract. Further, probiotics have the capacity to enhance gut barrier function and enhance local and systemic immunity through the effect on the human epithelium.

2.5.5 Antibiotic susceptibility test

Non-antibiotic resistance as a criterion for probiotics. Although most probiotics are known as Generally Regarded As Safe" (GRAS) due to their long history of safe use, their potential negative side effects are not zero. The presence of potentially transferable antibiotic resistance is a concern in the safety of probiotics. Therefore, it is essential to assess the safety of food products containing probiotics before they are marketed so that the risks do not outweigh its benefits. This method is essential for new strains of probiotics because it cannot be assumed that these newly identified probiotics share the same historical safety as traditional strains. Recently, many researchers have speculated that commensal bacteria may act as carriers of antibiotic resistance genes and transfer these genes to pathogenic or opportunistic bacteria. Probiotics that have been long considered to be non-infective have been isolated from lesions in patients with bacterial endocarditis and systemic infections. Therefore, probiotics that possess transmissible antibiotic resistance genes and the ability to translocate need further assessment before their use in food supplements (Mathur and Singh, 2006). The susceptibility of probiotics to antibiotics varies with the strain and its final application. Some probiotics are naturally sensitive to the majority of antibiotics, but others are naturally antibiotic-resistant. For example, *Leuconostoc* is naturally vancomycin-resistant, as are certain *lactobacilli* (Mathur and Singh, 2006). Probiotics can also be rendered multi-antibiotic-resistant by mutation or genetic modification. This method is essential for the pharmaceutical industry so that these probiotic scans survive oral antibiotic treatment. This probiotic antibiotic resistance would help prevent gastrointestinal side effects during antibiotic treatment (Courvalin, 2006). However, there is a drawback in this application: a higher risk of horizontal antibiotic resistance genes transfers from these multi-antibiotic-resistant probiotics to other gut pathogenic bacteria because antibiotic resistance genes acquired by the exogenous genes have a

greater possibility of gene transfer than the intrinsic chromosome-encoded genes or the resistance acquired through mutations, both of which are not transferable (Ammor, Florez and Mayo, 2007).

The Food and Agriculture Organization (FAO) and World Health Organization (WHO) have a report "Guidelines for the Evaluation of Probiotics in Food." This report outlined the recommended criteria and methodology for the evaluation of probiotics and the identification. The minimum requirements needed for probiotic status are studying the strain identity, the in vitro functional characterization to screen for the probiotic strain, the assessment of probiotic safety, and an in vivo study for efficacy (Bagchi, Lau and Ghosh, 2010). While probiotics are more documented for their gastrointestinal benefits, they could also be used to modulation of other health aspects such as the treatment and prevention of antibiotic resistance. Nevertheless, with new evidence showing that they could also harbor antibiotic resistance genes, more emphasis is given to the need for further assessment before consumption. It is now suggested that non-antibiotic resistance is set as a criterion for the safety assessment of probiotic strains meant for food applications (Smith and Jones, 2012). Resistances are not virulence factors by themselves, but infections with resistant microorganisms make difficult the course of the diseases. Once a *Lactobacillus* becomes resistant, the determinant is amplified and may be transmitted to another host. Therefore, checking for signs of transferable antibiotic resistance in probiotic bacteria used in the food is important. In contrast, antibiotic resistance of probiotic bacteria may be a desirable feature, as it could potentially help in their survival in the gastrointestinal tract, mainly when used after antibiotic therapy. (Salminen et al., 1998). The research showed that patterns of antibiotic susceptibility of the probiotic strains were quite variable. *L. plantarum* AF1 was found to be resistant to vancomycin (30 µg/disk), rifampicin (5 µg/disk), and streptomycin (10 µg/disk). It showed intermediate resistance to chloramphenicol (30 µg/disk), erythromycin (15 µg/disk), and penicillin (10 µg/disk). *L. plantarum* LS5 was resistant to vancomycin (30 µg/disk) and intermediately resistant to erythromycin (15 µg/disk). *L. paraplantarum* was resistant to streptomycin (10 µg/disk) and vancomycin (30 µg/disk) and showed intermediate resistance to erythromycin (15 µg/disk) and rifampicin (5 µg/disk). None of the tested probiotics were resistant to tetracycline (30 µg/disk) and ampicillin (10 µg/disk). Vancomycin (30

µg/disk) and streptomycin (10 µg/disk) resistance and sensitivity to rifampicin (5 µg/disk), penicillin (10 µg/disk), ampicillin 10 µg/ disk), and tetracycline (30 µg /disk) of *L. casei* 095, *L. casei* 032, *L. paracasei* RMS3-1, and *L. plantarum* WCFS1 isolated from traditional Kurdish cheese were reported. Intrinsic resistance of *Lactobacillus* strains to many antibiotics may be considered valuable for those isolates with probiotic potential. Such resistance could be appreciated for sustainable utilization of the strains in the human intestine to preserve the natural balance of intestinal microflora during antibiotic therapy of the host (Bacha, Mehari and Ashenafi, 2010).

2.5.6 Haemolytic activity assay

Haemolytic activities assay is one of the criteria for the safety of probiotic use. Haemolytic activities of the probiotic strains were recorded by the presence of beta (β) haemolysis indicated by colorless, a clear or lightened yellow zone surrounding the colonies representing total lysis of red blood cell. Alpha (α) haemolysis indicated by a small zone of greenish to brownish discoloration of the media, representing reduction of haemoglobin to methemoglobin which diffuses around, and gamma (ϒ) haemolysis with no change observed in the media (Reuben et al., 2019).

2.5.7 DNase test

A potential probiotic should be safe and non-pathogenic. For probiotics' safety, the strains were screened for DNase activities and none of the strains exhibited DNase activity. It means the selected isolates exhibit safe for consumption. All strains were tested on the DNase agar medium to check their production of DNase enzyme. Plates were incubated at 37 ◦C for 48 h. A clear, pinkish zone around the colonies after incubation was considered positive for DNase production (Gupta and Malik, 2007).

2.5.8 The 16S rDNA gene

The analysis of small rDNA gene sequences is another important landmark in studying the classification of living organisms. Traditionally, living organisms were classified, according to similarities and differences in their phenotypic characteristics, into prokaryotes and eukaryotes. Carl Woese and others started to analyze and sequence the 16S rDNA genes of various bacteria using DNA sequencing (Fox et al., 1977). In the last decade, sequencing of various bacterial genomes and comparison between genome and 16S rDNA gene phylogeny has confirmed the representativeness of the 16S rDNA gene in bacterial phylogeny (Snel, Bork and Huynen, 1999). The invention of PCR and

automated DNA sequencing two decades ago and subsequent work on 16S rDNA sequencing of bacteria, as well as 18S rDNA sequencing of eukaryotes, has led to the accumulation of a vast amount of sequence data on the rDNA genes of the smaller subunit of the ribosomes in a large number of living organisms. Comparison of these sequences has shown that the rDNA gene sequences are highly conserved within living organisms of the same genus and species. However, they differ between organisms of other genera and species.

Accurate identification of bacterial isolates is a fundamental task in clinical microbiology and provides insights into infectious disease etiologies and appropriate antibiotic treatment. Therefore, 16S rDNA sequencing represents a universal technology that, theoretically, provides solutions to these problems, yielding unambiguous data, even for unusual and slow-growing isolates, often within 48 h, reproducible among laboratories (Woo et al., 2008).

2.6 Prebiotics

This study examines some prebiotic properties of JA for screening and prepares to develop synbiotic powder of inulin and selected probiotic bacteria. Gibson and Roberfroid defined a prebiotic as "a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and activity of one or a limited number of bacteria in the colon, and thus improves host health." The concept of prebiotics arose from the observation that inulin and fructooligosaccharides (FOS) selectively stimulate the growth of bifidobacteria, which are considered beneficial for human health (Roberfroid et al., 2010). Table 2.3 shows types and sources of prebiotics.

A wide variety of dietary carbohydrates, mainly resistant starch, dietary fibers, and non-digestible oligosaccharides, have such characteristics, and they provide available substrates for bacterial fermentation in the colon. The colonic fermentation plays a role in salvaging part of their energy, controlling transit time, stool bulking and stool frequency, influencing nutrient mainly mineral, bioavailability, producing short chain fatty acids that are known to play physiological roles such as control of mucosal motility and epithelial cell proliferation or in modulating immune activity and endocrine functions (Roberfroid et al., 2010) (Figure 2.3).

Table 2.3 Types and sources of prebiotics

Type	Sources
Fructooligosaccharides	Asparagus, sugar beet, garlic, chicory, onion, Jerusalem artichoke, wheat, honey, banana, barley, tomato, and rye
Isomaltulose	Honey, sugarcane juice
Xylooligosaccharides	Bamboo shoots, fruits, vegetables, milk, honey, and wheat bran
Galactooligosaccharides	Human's milk and cow's milk
Soybean oligosaccharide	Soybean
Lactulose	Lactose (milk)
Lactosucrose	Lactose
Maltooligosaccharides	Starch
Palatinose	Sucrose
Isomaltooligosaccharides	Starch
Enzyme-resistant dextrin	Potato starch

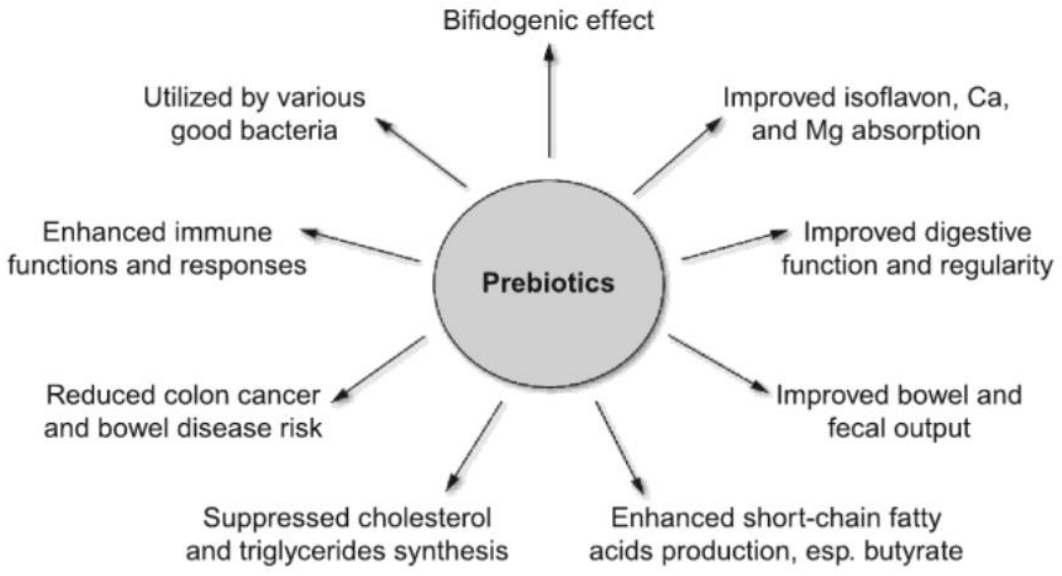


Figure 2.3 Some proposed health benefits of prebiotics

Source: Watson and Preedy (2015)

In the industrialized world, there has been an explosion of consumer interest in the active role of foods in the well-being and life prolongation as well as in the prevention of initiation, promotion and, development of cancer, cardiovascular diseases, and osteoporosis (Grajek, Olejnik and Sip, 2005). Therefore, a new term functional food was proposed (Roberfroid, 2007). Food can be regarded as functional if it is satisfactorily demonstrated to affect beneficially one or more target functions in the body (Roberfroid, 2002). Among the most promising targets for functional foods are the gastrointestinal functions, including those that control the transit time, bowel habits, mucosal motility, and those that modulate epithelial cell proliferation (M B Roberfroid, 2000). At the moment, prebiotic is one of the most important and frequently used compounds in functional food (Table 2.4). On the contrary, the functional food industry's perception of the importance of gut microbiology in human health and nutrition has led to a significant increase in prebiotic and prebiotic-based products (Gibson, 2008). At present, the majority of prebiotic oligosaccharides are produced on an industrial scale and are widely available on the market (Grajek, Olejnik and Sip, 2005).

As with any food component, the question of a prebiotic's safety still needs to be considered. To determine whether inulin/oligofructose prebiotics are safe at increased levels of intake, critical review and evaluation of the animal toxicological data are undertaken by Carabin and Flamm (Carabin and Flamm, 1999). According to their review, the studies have demonstrated that inulin-type prebiotic fructans, when administered in the diet at high levels, does not result in mortality, morbidity, target organ toxicity, reproductive or developmental toxicity, or carcinogenicity. Several in vitro studies have also shown the absence of mutagenic or genotoxic potential. On the contrary, the oligofructose evaluated demonstrated an excellent safety profile, both in a 13-week in vivo and in vitro analyses (Boyle et al., 2008).

Table 2.4 Prebiotic applications as a functional food

Applications	Functional properties
Yogurts and desserts	Sugar replacement, texture and mouthfeel, fiber, and prebiotics
Beverages and drinks	Sugar replacement, mouthfeel, foam stabilization, and prebiotics
Breads and fillings	Fat or sugar replacement, texture, fiber, and prebiotics
Meat products	Fat replacement, texture, stability and fiber
Cake and biscuits	Sugar replacement, moisture retention, fiber, and prebiotics
Chocolate	Sugar replacement, heat resistance and fiber
Sugar confectionary	Sugar replacement, fiber, and prebiotics
Soups and sauces	Sugar replacement, and prebiotics
Baby food	Texture, body and mouthfeel, fiber, stability, and prebiotics

Source: Faria et al. (2011)

It is known that the safety of traditional food is commonly not questioned, i.e., they are considered safe, as we have a long history of experience. In the evaluation of prebiotics, this experience with the prebiotic compounds is of paramount importance and provides us with confidence in its safety. It is clear that overeating fructooligosaccharide causes discomfort and, thus, their presence in all foods may be quite undesirable. Therefore, the only basis for limiting the use of such prebiotic in the human diet relates to gastrointestinal tolerance. Most prebiotics is marketed as foodstuffs, and therefore the consideration of the safety of prebiotics is of utmost importance. The health benefits of prebiotics are associated with the gastrointestinal microflora, especially lactic acid bacteria and *bifidobacteria*. However, many bacteria of the genera *Lactobacillus*, *Bifidobacterium, Pediococcus, Enterococcus, Leuconostoc*, and were isolated frequently from various types of infective lesions (Ishibashi and Yamazaki, 2001). Thus, the safety consideration of prebiotics used industrially and commercially is essential, although no direct evidence shows the adverse effect of

prebiotics on human health. Besides, some microbial species in our gastrointestinal tract, such as the genus Enterococcus are considered opportunistic pathogens. The relation between the prebiotics and these kinds of bacteria, or the possibility of strain specific infection, requires further studies.

The development of prebiotics is a multidisciplinary effort. Its future success depends on effective cooperation requiring new ideas from food technologists, strong clinical support from medical and nutrition scientists, and understanding current and future needs in food and human health consumer information experts. It is still early days for prebiotics, but they offer the potential to modify the microbial balance in the gut in such a way as to bring direct health benefits cheaply and safely. They can be incorporated into many foodstuffs. There are, however, long-term, double-blind, randomized placebo-controlled human intervention studies that still need to be carried out in different populations to obtain consistent evidence about the beneficial effects of prebiotics on human health. Indeed, the studies related to applications of prebiotics from various angles are not a simple task.

2.7 Inulin

Inulin, a naturally occurring polysaccharide, belongs to a class of dietary fibers known as fructans. The term fructans are generally used for those compounds in which fructosyl moieties constitute the molecule. Inulin, a peculiar substance, was first isolated from Inulahelenium by a German scientist, Rose (1804), and later on, it was named inulin by Thomson (1817). In nature, it is the second most abundant storage polysaccharide. Structurally inulin [α-D-glucopyranosyl-β-D-fructofur-anosyl-(n-1)-D-fructofuranoside] is composed of β-D (2→1) linked fructosyl oligomer with a glucose moiety at the reducing end. Glucose unit joined by α-D (1→2) glycosidic bond is present in pyranose form (4C1conformation), whereas the fructose unit is in the furanose form. Generally, the inulin has a glucose moiety at the reducing end, but in some cases, it has only fructose molecules lacking the glucose at the terminal end (Meyer and Blaauwhoed, 2009). The unique aspect of the inulin structure is that no bond of its fructose ring is part of the macro-molecular backbone (André et al., 1996). Inulin was designated a GRAS (Generally Recognised As Safe) status since 2002 because of its use in food industries, including meat and poultry products and baby products. In many

countries, inulin-rich plants are used as an essential part of the regular diet. An average daily intake of inulin in Western and American diets has been estimated to 1–10 g and 2.5 g, respectively. However, in the European diet, inulin consumption is relatively higher (3–11g) per day. Inulin has many health-promoting properties, due to which it is considered a functional food. Owing to its high degree of polymerization (DP), it acts as a potential prebiotic in food processing industries (Ram S Singh and Singh, 2010). Inulin, in combination with FOSs (fructooligosaccharides), is used as a non-digestible dietary fiber that promotes the human gut microbiota by stimulating the growth of *bifidobacteria* in human intestine that beneficially affects the host's body. Apart from its prebiotics effect, it is also used in the lipid metabolism, absorption of mineral ions from the gut, control of blood sugar level and prevention of obesity, chemically induced aberrant crypts, colon cancer. (S. Singh and Singh, 2010).

A lot of inulin-rich plants are reported from both monocots and dicotyledonous families. Inulin is present in a considerable amount in bulbs, tubers, and tuberous roots of many plants like *Dahlia pinnata* (Dahlia), *Taraxacum officinale* (Dandelion), *Asparagus officinalis* (Shatwaar), *Helianthus tuberosus* (Jerusalem artichoke), *Asparagus racemosus* (Safed musli), *Cichorium intybus* (chicory). (Ram S Singh and Singh, 2010) (Table 2.5). Therefore, such inulin-rich feedstocks are of great consideration as they are an inexpensive, renewable, and abundant substrate for the production of various bioproducts. Inulin-rich plant materials and mixed substrates can be used as potent substrates for various bio-processes. Inulin-rich feedstocks have been extensively used for the production of high fructose syrup, inulinases, inulooligosaccharides, biofuels, organic acids, single cell oil, single cell proteins, mannitol, sorbitol, 2, 3 - butanediol, pullulan, etc. The utilization of inulin-rich feedstocks for the production of high fructose syrup, inulinases, fructooligosaccharides, biofuels, organic acids, etc has been reviewed (Singh et al., 2019).

Table 2.5 Inulin content of various inulin containing plants

Common name	Botanical name	Plant part	Inulin content (%)*
Agave	*Agave americana*	Lobes	7-10
Artichoke	*Cynara cardunculus*	Leaves-heart	3-10
Banana	*Musa acuminata*	Fruit	0.3-0.7
Barley	*Hordeum vulgare*	Grains	NS
Burdock	*Arctium sp.*	Roots	3.5-4.0
Camas	*Camassia sp.*	Bulbs	12-22
Chicory	*Cichorium intybus*	Roots	15-20
Dahlia	*Dahlia sp.*	Root tubers	15-20
Dandelion	*Taraxacum officinale*	Leaves	12-15
Garlic	*Allium sativum*	Bulbs	9-16
Jerusalem artichoke	*Helianthus tuberosus*	Roots	12-19
Kuth	*Saussurea lappa*	Roots	18-20
Lettuce	*Lactuca sativa*	Roots	NS
Murnong	*Microseris lanceolata*	Roots	8-13
Onion	*Allium cepa*	Bulbs	2-6
Safed musli/shatwaar	*Asparagus racemosus*	Root tubers	10-15
Salsify	*Tragopogon* sp.	Roots	15-20
Shatwaar	*Asparagus officinalis*	Root tubers	10-15
Spanish salsify	*Scorzonera hispanica*	Roots	8.15-10.75
Suma	*Pfalia glomerate*	Roots	11.45*
Sunflower	*Helianthus annuus*	Root tubers	NS
Sweet leaf	*Stevia rebaudiana*	Roots	18-23*
Yacon	*Smallanthus sonchifolius*	Roots	3-19

Source: Singh et al. (2019)

Note: NS: Not specified, *Percentage of fresh mass, A percentage of dried mass

Effect of inulin and FOS on the gastrointestinal microbial community. Dietary supplementation of inulin-type fructans is classified as non-digestible food ingredients that beneficially affect the host by stimulating growth and activity of a limited number of health-promoting bacteria such as *Lactobacillus* and *Bifidobacteria* in the intestine, and thus improves host health (Gibson and Roberfroid, 1995). Probiotic bacteria selectively ferment inulin and FOS to produce SCFA (acetate, propionate, and butyrate). Reducing the pH of the colon resulting from the production of SCFA is another prebiotic property. Lower pH values inhibit the growth of certain pathogenic bacterial species while stimulating the growth of the *bifidobacteria* and other lactic acid species (Mussatto and Mancilha, 2007).

Inulin is a natural storage polysaccharide with a large variety of food and pharmaceutical applications. It is widely distributed in a plant, being present as a storage carbohydrate in more than 30,000 vegetable products. Due to their wide distribution in nature and significant role in the industry, the extraction, isolation, and characterization of inulin-type fructans are gaining attention in recent years. Inulin sources have recently received increasing interest because they are a renewable raw material for the production of bioethanol, fructose syrup, single-cell protein, and single-cell oil, and obtainment of FOS and other useful products.

Fructans are one of the most widely sued prebiotic. Inulin-type fructans are commonly commercially produced from chicory roots (*Cichorium intybus* L.) or Jerusalem artichoke tubers (*Helianthus tuberosus*). Inulin is a very interesting functional ingredient that is increasingly being used to formulate new fiber-enriched products or with prebiotic benefits (Roberfroid et al., 2010). Chemically, it is a polymer of fructose units that can have different chain lengths. Long-chain inulin can modify texture because it crystallizes in water, forming a particle network structure.

Consequently, it is being used in low-fat products to improve creaminess and consistency, mimicking full-fat products. Nutritional studies have recommended combining inulin with different chain lengths to maximize its fermentative and prebiotic effects. From a technological viewpoint, mixed-chain inulin, instead of only long-chain, may decrease its thickening ability. However, this effect can also depend on inulin concentration and its possible interaction with other ingredients and their concentrations.

Jerusalem artichoke (JA) (*Helianthus tuberosus* L.) or Kaentawan. It is a native plant of North America. It is presently cultivated in Europe, Australia, and Asia (Baldini et al., 2004). In Thailand, Kaentawan can be harvested after 90-120 days, allowing a chance for farmers to produce 3 crops per year and crop yields of Kaentawan are typically 13-19 tons per hectare. The tubers of Kaentawan (Figure 2.4) generally contain about 80% water, 15% carbohydrate, and 1-2% protein. Its tuber contains a high amount of dietary fiber inulin and FOS (15.28 and 5.96 g/ 100 g fresh weight, respectively). The tubers contain 68-83% fructans, 1.5-1.6% proteins, 13% insoluble fiber and 5% ash on a dry weight loss. Interestingly, Kaentawan tubers do not contain starch. It has traditionally been used as food and animal feed and, more recently, as a raw material for the industrial production of fructose and fructans.

Figure 2.4 Jerusalem artichoke tubers

Source: Medthai.com (2019: Website)

Generally, inulin and FOS are natural food components belonging to a class of carbohydrates knowns as fructans. The Kaentawan tubers accumulate high fructans (68-83% on dry weight) during their growth (Fleming, GrootWassink and Murray, 1979). Inulin and FOS are fructans that are not hydrolyzed by pancreatic enzymes and escape digestion in the small intestine. Beneficial bacteria, especially *Bifidobacteria*, have relatively high amounts of 13-fructosidase that are selective for [3 2-1 glycosidic bonds in fructans. After oligosaccharide hydrolysis, monomers then serve as a suitable growth substrate for the bifidus pathway of hexose fermentation. Fructan containing foods were reported to enhance mineral absorption, reduce cholesterol levels, stimulate

the body's immune system, and decrease the levels of pathogenic bacteria in the intestine (Kaur and Gupta, 2002).

2.8 Synbiotics

A product denoted as a synbiotic is one in which a probiotic microorganism and prebiotic ingredient are combined. An additional required condition to be fulfilled for synbiotic foods is that the chosen prebiotic must selectively support the growth of the probiotic microorganism employed.

Pro - and prebiotics are often combined in a food product resulting in a synbiotic, which is defined as "a mixture of a probiotic and a prebiotic that beneficially affects the host by improving the survival and the implantation of live microbial dietary supplements in the gastrointestinal tract, by selectively stimulating the growth and by activating the metabolism of one or a limited number of health-promoting bacteria, and thus improving host welfare." Pro- and prebiotics are intended to modify the gastrointestinal microflora in such a way that bacterial activities advantageous to the host are stimulated, and those averse to host health are suppressed. It is reasonable to assume that probiotics and prebiotics, respectively, have preferred sites of action in the gut. Probiotics act mostly in the small intestine and prebiotics, usually in the colon. The human GIT is a complex microbial ecosystem composed of at least 10^{14} bacterial cells, a number that exceeds the total human body cells by a factor of ten. The bacteria in the GIT are not evenly distributed, and their diversity and numerical importance vary in the different sections of the GIT. Whereas the stomach and duodenum are only sparsely populated with 10^{2}-10^{5} cells of mostly facultative anaerobic bacteria per milliliter, the lower distal part of the human gut harbors a large variety of mostly anaerobic species, belonging to the genera *Bacteroides, Bifidobacterium, Fusobacterium, Eubacterium, Peptostreptococcus, Clostridium,* and *Ruminococcus.* Facultative anaerobic bacteria make up a minor proportion of the human colonic flora, which in essence, is reflected by the faecal flora. The cell density of colonic contents may reach 10^{12} bacterial cells/g dry mass (Pandey, Naik and Vakil, 2015).

For the development of Synbiotics as previously stated, the definition of prebiotic was recently restructured to "a substrate that is selectively utilized by host microorganisms conferring a health benefit. Hence, the definition is not limited only to

fermentable carbohydrates but can also include polyphenols and polyunsaturated fatty acids. For instance, polyphenols might reach the colon and be further metabolized by gut microbiota. Prebiotic compounds were focused on conveying a health effect in the gut, reduced cardiometabolic risk, and mental health (e.g., enhanced cognition). However, the new definition includes the modulation of any host microbial ecosystem. Also, prebiotics will target possible health effects by modulating populations of *Roseburia, Eubacterium* and *Faecalibacterium* spp. populations apart from *Lactobacillus* and *Bifidobacterium.*

Fructans, FOS and inulin, and galacto-oligosaccharides (GOS) and lactulose are the most commonly used compounds as prebiotics. Nonetheless, an ample range of carbohydrates with different monosaccharide content and configuration of glycosidic linkages could exhibit the potential prebiotic effect. Novel prebiotic compounds could derive from natural and renewable resources or be synthesized enzymatically. Dietary fibers and their hydrolysis products are becoming an emerging source of new ingredients with potential prebiotic activity.

Implementation of "-omic" approaches could elucidate underlying mechanisms of interaction between gut microbiota, including cross-feeding phenomena along with selectivity and specificity on fermentable carbohydrates and other components. Hence, the use of prebiotics is gaining considerable interest, as they target to sustain a healthy microbiome or restore microbial dysbiosis.

A synbiotic includes a probiotic and a prebiotic combination and should target to enhance the survival and implantation of the probiotic in the GIT to promote beneficial bacterial groups (Figure 2.5). Following the definition, a synbiotic can have either complementary or synergistic action. In the first case, the prebiotic is independently selected to enhance indigenous beneficial microbiota, and the probiotic is selected for targeted biological action. On the other hand, in the latter case, the prebiotic is chosen to support the selected probiotic's growth precisely. Hence, the prebiotic is included to be selectively fermented by the probiotic strain, regardless of the beneficial impact on the population of other bacteria. The development of synbiotics is emerging to be of paramount importance as it can be used as a supplement in food and nutraceutical applications. Nutraceuticals can be used as dietary supplements or functional foods, as they are food ingredients or sourced from food products that, apart from the primary

original nutritional value, provide extra benefits (e.g., chronic disease prevention, improving health) **(**Figure 2.5).

Synbiotics, from a technological viewpoint, were also designed to overcome difficulties such as cell Survival in the GIT. Likewise, the development of a combination in a single product could ensure an effective formulation compared the probiotic or prebiotic activity alone. Features like long-term stability during the shelf-life of food, drinks, and resistance of probiotics to processing also exhibit a positive effect on the use of synbiotic. Most synbiotic formulations include either yogurts or dairy drinks. However, new products are under ongoing design. For instance, the development of synbiotic milk chocolate using encapsulated *L. casei* cells has been reported by Mandal et al. Milk chocolates presented a promising food the delivery system for probiotics, whereby cell viability was enhanced with inulin. Studies conducted in vivo in mice fed with synbiotic milk chocolate led to an increase in faecal lactobacilli, decreased Faecal coliforms and β-glucuronidase activity. Criscio et al. developed prebiotic, probiotic and synbiotic ice creams, whereby the synbiotic was formulated with inulin and *Lactobacillus* strains. Viable counts to ensure probiotic dosage were documented after frozen storage, where organoleptic characteristics were also maintained. Encapsulation of food bioactive in micro- and nanoparticles via nanoscale control of food molecules could modify and enhance desired characteristics to develop functional foods. Encapsulation approaches aiming to develop synbiotics have already been applied in the literature. Other novel approaches have also been presented, such as non-dairy synbiotic beverages. In that study, selected probiotic LAB were encapsulated by incorporating into rice-berry malt extract (RME). The aim was the production of a lactose-free product through alginate hydrogel encapsulation, including inulin. The results were quite promising since the RME medium supported the selected LAB and alginate hydrogel growth significantly improved their survivalbility in the GIT. Finally, the authors suggested that the synbiotic beverage maintained high concentrations of *L. plantarum* cells under cool storage for two weeks. Co-encapsulation of synbiotics usually occurs by employing alginate as a matrix. Alginate gels are stable at low pH values and can be swelled at higher pH values (as in intestinal environments) whereby the release of the cells enhanced viability. Atia et al., studied an alginate-inulin synbiotic co-encapsulation of probiotic to target delivery in the colon as a site action. The results revealed that

formulations containing inulin improved muco-adhesion properties of the probiotic beads, also increasing their protection from the acidic environment. More recently, synbiotic encapsulation of *L. plantarum* was also evaluated, using alginate-arabinoxylan composite microspheres, whereby encapsulation efficiency, along with survival and storage stability, were enhanced. The same probiotic species have also been studied by Vaziri et al. applying co-microencapsulation with DHA fa.

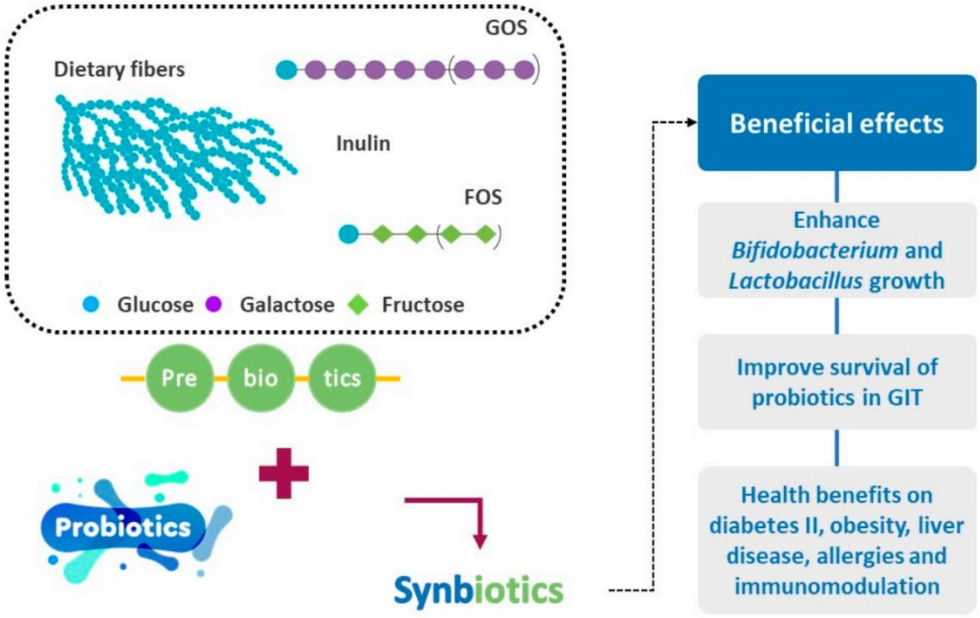


Figure 2.5 Beneficial effects of synbiotics

Source: Terpou et al. (2019)

2.9 Freeze drying method

During the manufacturing process of probiotic food, beneficial microbes encounter various stresses. Because the microbial inoculation into the carrier food should ensure a high load of microbes into the final product, throughout its shelf life, probiotics are first grown to high numbers, on an industrial scale, using food-grade culture media. As microbial inocula are usually stored as frozen stock the major stress derives from low temperature and temperature changes at this stage. Following the phase of biomass production, two main alternative drying techniques (spray drying and freeze-drying) can be used to generate high cell density probiotic powders, which can then be added to several types of food (Peighambardoust, Tafti and Hesari, 2011).

Freeze-drying requires milder conditions and is generally better tolerated by bacteria. During such a process, exposure to very low temperature and dehydration represent the main sources of stress. Such conditions cause alterations of cell membrane integrity and fluidity (Schwab, Vogel and Gänzle, 2007), protein denaturation, enzyme inactivation, and DNA damage, with detrimental effects on microbial metabolism and viability. During the following storage of dried probiotic biomasses, loss of viability and activity is quite common. This is due to the combined stress of temperature changes, phase changes, and drying, which causes damage mainly to the cell membrane and associated proteins.

Probiotics are most commonly used as foods in the form of fermented milk products or yogurt. Consequently, they require refrigeration and their shelf life is limited to only a few weeks, causing logistical problems for manufacturers and inconvenience for consumers. This also makes probiotics very unsuitable for travelers who rely on probiotic bacteria to prevent travelers' diarrhea. As a solution to this problem, probiotics have been developed into freeze-dried solid forms that have a longer shelf life and do not need refrigeration (Otero, Espeche and Nader-Macías, 2007) thus making them more convenient for both manufacturers and consumers alike. The freeze-drying process involves a solvent, typically water, being removed from a frozen solution via sublimation. However, freeze-drying can lead to cell injury and decreased viability due to the exposure of cells to the attenuating effects of freezing and dehydration. Loss of viability during freeze-drying is due to temperature changes, phase changes and dehydration, resulting in damage to cell membranes, cell walls, ribosomes and DNA (Semyonov, Ramon and Shimoni, 2011). Despite this, freeze-drying is still gentler than other techniques such as spray drying, permilling higher probiotic survival rates. Probiotic viability can be enhanced during freeze-drying by selecting specific strains, which exhibit higher survival rates than others. Also, several factors have been identified as critical to cell survival, including initial cell mass, growth conditions, the composition of growth and drying media, and rehydration conditions. Freeze-dried probiotics can be divided into two categories, those that are nonencapsulated and those that are encapsulated (Liong, 2011).

Probiotic bacteria can produce a wide range of metabolites with health benefits to humans. Bioactive compounds produced by probiotic bacteria include, e.g.,

bacteriocins, metabolic enzymes, amino acids and peptides, short-chain in fatty acids, vitamins, antioxidants, anti-inflammatory and immune-modulating agents, and exopolysaccharides. Collectively, these molecules enhance the physiological function of the gut and improve health. The most important criteria for supplementation of foods with probiotic microorganisms relates to maintaining the viability of the microbial cells during the processing, packaging, and storage of the food. Labelling food as a probiotic functional food must contain the minimum therapeutic level of viable probiotic microorganisms throughout the product shelf-life, i.e., at least 10^6 CFU/g. It is suggested that a daily intake of 10^8–10^9 CFU probiotic bacteria per gram food is necessary to cause the positive physiological functions of humans. Thus, ~100 gram/day of probiotic products should be consumed daily in order to deliver the required number of probiotic cells to the human body. The viability of probiotic cells is affected by a wide range of factors that can be classified as chemical (pH, water activity, and prebiotics), biological (probiotic strain, symbiosis/antagonism with starter cultures and product natural microflora, and occurrence of pathogenic or spoilage microorganisms), and physical (mainly temperature and oxygen availability. Processing strategies based on drying techniques (freeze-drying, spray-drying, and vacuum-drying) and encapsulation techniques are currently being investigated as a means to maintain probiotic cell viability and functionality. The symbiosis between probiotic bacteria and prebiotics, defined as substrates that confer health benefits by significantly affecting the viability of the probiotic bacterial cells. During formulation and processing, probiotic bacteria should be able to withstand and adapt to several stress factors, e.g., oxygen, acid, salt, and heat. Encapsulation in polysaccharides, proteins, or gums is a dominant strategy to enhance the viability of probiotic bacteria. Thus, probiotics exist as live microorganisms and freeze-dried powders formulated as capsules, powders, or tablets. Besides maintaining the viability of the microorganism (s) during food processing, packaging, and storage, other important aspects include the safety of the microorganism, its ability to colonize in the intestinal epithelium, its antimicrobial activity against pathogenic strains, its effects on the immune system, and its overall benefits. (Chugh and Kamal-Eldin, 2020).

2.10 Gastrointestinal physiology

The digestive system is directly related to the probiotic study in which the human digestive system origin from the mouth to the anus, so probiotic studies for health benefits need to understand the function of this system. Anatomically and physiologically, the digestive system is divided into the tubular gastrointestinal tract (GIT) and accessory digestive organs (Figure 2.6). The GIT is approximately 6 m in length and extends from the mouth to the anus. The organs of GIT comprise oral cavity, pharynx, esophagus, stomach, small intestine, and large intestine. The accessory digestive organs are the teeth, tongue, and salivary glands, liver, gallbladder, and pancreas (Silva and Freitas, 2014).

The GIT wall is composed of four layers: from the outer surface to the inner surface are the serosa, muscularis, submucosa, and mucosa. The mucosa is composed of a simple columnar epithelium separating the highly colonized intestinal lumen from the second, underlying layer, the lamina propria, and the muscularis mucosa.

A mucus gel layer covers a large part of the GIT epithelium. The mucosa is the absorptive layer, maximizing nutrient absorption, and has secretory and barrier function preventing the passage of strange luminal components (bacteria, food components), thus inhibiting pro-inflammatory host response. Adjacent intestinal epithelial cells form tight junctions, which help maintain such selective impermeable barrier function. Paneth cells and goblet cells also contribute to this function via innate immune defenses. Paneth cells are responsible for the production of several antimicrobial compounds, including defensins and lysozyme, which prevent interaction between microorganisms and proliferative cells in the crypts.

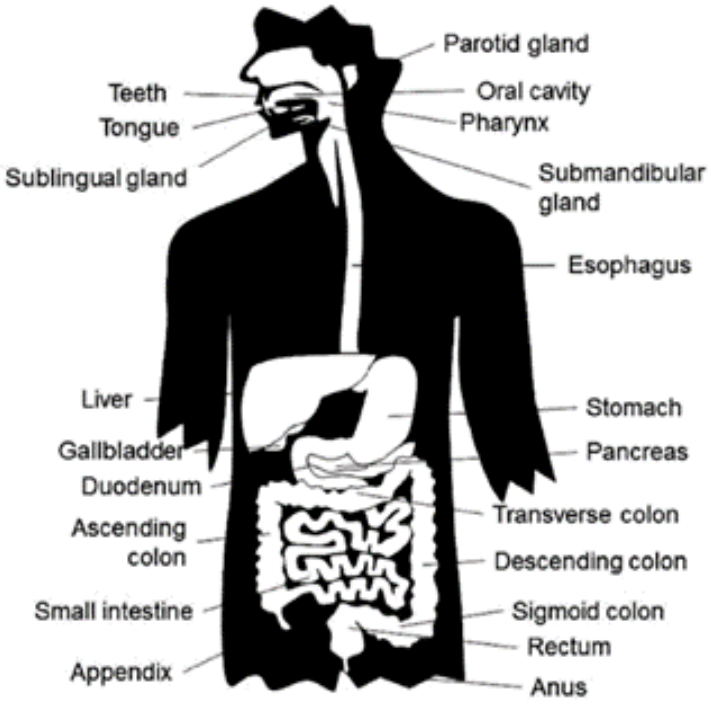


Figure 2.6 Anatomic structure of the human GIT and accessory digestive organs
Source: Silva and Freitas (2014)

In contrast, goblet cells produce mucins, which confer to the epithelium its barrier effect by preventing direct contact from luminal microorganisms. M cells or microfold cells founded in lymphoid tissue of ileum (Peyer's patches) are involved in the absorption of macromolecules, particularly protein antigens. The lamina propria is a special type of essentially sterile connective tissue that contains lymph nodules, which are involved in the protection against disease. The muscularis mucosa is responsible for the small folds in some parts of GIT (Silva and Freitas, 2014).

Among other factors such as transit time, bacteria metabolism, and chemical reactions that might influence the performance of probiotic products, the pH is perhaps the most important due to the general sensibility of probiotics to acidic conditions. The average pH in the stomach is 1.8 and rises to 6.6 in the proximal intestine reaching 7.5 in the distal intestine, and then it decreases to 6.3 in the right colon. It rises again until it reaches 7.1 in the left colon (Evans *et al.*, 1988). The pH depends on the prandial condition of individuals. Apart from the prandial condition, the pH in GIT is a function

of many variables, including meal volume and content, and volume of secretions. It should be noted that the extrapolation of GIT pH data from healthy situations to pathological conditions must be made with precaution. Table 2.6 shows physiological pH in the GIT in the fasted and fed states.

Table 2.6 Physiological pH in the GIT in the fasted and fed states

Gastrointestinal site	Fasting	Fed
Stomach	1.4 - 2.1	3.0 - 7.0
Duodenum	4.9 - 6.4	5.1 - 5.2
Jejunum	4.4 - 6.5	5.2 - 6.2
Ileum	6.5 - 8.0	6.8 - 8.0

Source: Silva and Freitas (2014)

Thus, it is not surprising that GIT pH varies between and within individuals. The transit time of a probiotic product in the intestine is also a determinant of the viability of probiotics, as the exposure to the harsh conditions will depend substantially on gastric residence time. The oesophageal residence time is usually short and the small intestine residence time is relatively constant (3-4 h). However, the gastric time varies widely (5 min to 2 h, although much longer times have been reported) according to factors such as volume, osmolality, pH, viscosity, and nature of ingested material or even emotional factors and age. Thus, the total residence time depend on gastric emptying rate and flow rate and can exhibit a significant intraindividual and interindividual variability (Mudie, Amidon and Amidon, 2010).

In Mouth and Oesophagus, during mastication or chewing, the contact of food and other products with oral mucosa is generally brief but sufficient to initiate the digestion of the starch by the salivary amylase. After this rapid passage through the mouth, the swallowed food is driven to the stomach by the peristaltic movements of the esophagus. The esophagus is a muscular tube that connects the pharynx to the stomach and has a lumen pH between 5 and 6. The oesophageal transit time for dosage forms, liquids, or boluses of solids is approximately 10-20 s.

The stomach has a J-shape with an approximate capacity of 1.5 1, but it usually contains 20-30 ml of fluid in a fasting state. The stomach acts as a reservoir for food, while it is mixed with acid, mucus, and pepsin in order to be released into the duodenum at a controlled rate. The gastric emptying time may vary from a few minutes to several hours, depending on the time of the last food ingestion, anxiety, position, and level of individual activity, among other factors.

During fasting, an interdigestive cycle of motility, this cycle begins in the stomach and moves along the small intestine into the distal ileum a burst of contractile activity of 5-15 min opens the pylorus and clears the stomach. The ingestion of food stops the cycle.

The peristaltic movements appear 5-10 min after the intake of foods and remain until the gastric emptying, which lasts from 1 to several hours, depending on the meal composition. The hydrochloric acid secreted by parietal cells kills many bacteria and provides the pH for pepsin to begin protein digestion (Ganong, 2005).

The small intestine extending from the pyloric sphincter of the stomach to the ileocaecal valve has three distinct parts, namely, duodenum (20-30 cm), jejunum (approx. 2 m), and ileum (approx. 3 m). The main functions of this organ are the digestion of foods and the absorption of nutrients and other materials.

In the lumen of the small intestine, foods and other products are mixed with the bicarbonate (from Brunner's glands, located in the duodenum), secretions of mucosal cells (mucus and enzymes), pancreatic juice (sodium bicarbonate and enzymes), and bile (bile acids, phospholipids, and bilirubin). The detergent property of bile confers a potent antimicrobial activity that hampers the survival of the microorganisms (including many probiotics) in the GIT. The small bowel constitutes a transition zone between stomach bacterial scarcity and highly populated colon.

The large intestine stretches from the ileocecal valve to the anus and has two main functions: the absorption of water, sodium, and chloride ions and the storage of feces. The colon is composed of the caecum, the ascending colon, the transverse colon, the descending colon, the sigmoid colon, and the rectum. The slower colon motility is responsible for transit times up to 60 h, which undoubtedly contributes to the tremendous number of microorganisms found in the colon. The large intestine is colonized by about 10^{12} bacteria per Gram of intestinal contents (about 35-50% of the

volume content of the colon), which are responsible for several metabolic reactions. These microorganisms may be in the lumen, mucus gel, or mucosal of epithelial cells. The equilibrium of the microbial groups present in the GIT is essential for human health. *Bifidobacterium* and *Lactobacillus* species are the main strains with identified beneficial properties in the indigenous GIT microflora (Silva and Freitas, 2014).

CHAPTER 3

MATERIALS AND METHODS

3.1 Media

3.1.1 Agar powder, HiMedia, India

3.1.2 Columbia Blood Agar, HiMedia, India

3.1.3 Deoxyribonuclease (DNase) Agar, HiMedia, India

3.1.4 Dulbecco' Modified Eagle Medium (DMEM), Sigma-Aldrich, USA

3.1.5 de Man, Rogosa and Sharpe (MRS) Agar, HiMedia, India

3.1.6 de Man, Rogosa and Sharpe (MRS) Broth, HiMedia, India

3.1.7 MacConkey Agar, HiMedia, India

3.1.8 Mannitol Salt Agar (MSA), HiMedia, India

3.1.9 Mueller Hinton Agar (MHA), HiMedia, India

3.1.10 Phenol Red Egg Yolk Polymyxin (MYP) Agar Base, HiMedia, India

3.1.11 Tryptic Soy Broth (TSB), HiMedia, India

3.1.12 Salmonella-Shigella Agar (SSA), HiMedia, India

3.2 Chemical reagents

3.2.1 Alpha-amylase from human saliva, Sigma-Aldrich, USA

3.2.2 Bile salt, Hyclone, USA

3.2.3 Fetal bovine serum, Hyclone, USA

3.2.4 Glycerol, Sigma-Aldrich, USA

3.2.5 Gram stain, Sigma-Aldrich, USA

3.2.5.1 Crystal violet solution

3.2.5.2 Decolorizer solution

3.2.5.3 Gram's iodine solution

3.2.5.4 Safranin solution

3.2.6 Hydrochloric acid (HCl), Sigma-Aldrich, USA

3.2.7 Methanol, Sigma-Aldrich, USA

3.2.8 Oxbile (oxgall) powder, Sigma-Aldrich, USA

3.2.9 Oxytetracycline 900 µg, Sigma-Aldrich, USA

3.2.10 Pancreatin, Hyclone, USA

3.2.11 Penicllin-streptomycin, Hyclone, USA

3.2.12 Pepsin, Hyclone, USA

3.2.13 Phosphate Buffered Saline PBS pH 7.4(1X); gibco, Sweden

3.2.14 Potassium dihydrogen phosphate (KH_2PO_4), Sigma-Aldrich, USA

3.2.15 Serum-human venous blood, Hyclone, USA

3.2.16 Skim milk, HiMedia, India

3.2.17 Sodium hydroxide (NaOH), Sigma-Aldrich, USA

3.2.18 Sodium phosphate dibasic dehydrate ($Na_2HPO_4.2H_2O$), Sigma-Aldrich, USA

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

3.2.20 Tetracycline Hydrochloride, Applichem Panreac; Germany

3.2.21 Triton X-100, Merch, Germany

3.2.22 Trypan blue, Sigma-Aldrich, USA

3.2.23 Trypsin EDTA, Hyclone, USA

3.3 Antibiotics disc

3.3.1 Ampicillin (10 µg), Applichem Panreac; Germany

3.3.2 Gentamicin (10 µg), Applichem Panreac; Germany

3.3.3 Norfloxacin (10 µg), Applichem Panreac; Germany

3.3.4 Sulphamethoxazole (25 µg), Applichem Panreac; Germany

3.3.5 Tetracycline (30 µg), Applichem Panreac; Germany

3.4 Microorganisms

3.4.1 Bacterial indicators

3.4.1.1 *Bacillus cereus* DMST 5040

3.4.1.2 *Escherichia coli* ATCC 25922

3.4.1.3 *Pseudomonas aeruginosa* DMST 4739

3.4.1.4 *Salmonella enteritidis* DMST 15676

3.4.1.5 *Salmonella typhimurium* DMST 560

3.4.1.6 *Staphylococcus aureus* ATCC 25923

3.4.2 Reference strains

3.4.2.1 *Lactobacillus casei* TISTR 1341

3.4.2.2 *Lactobacillus plantarum* TISTR 541

3.4.2.3 *Staphylococcus epidermidis* DMST 15505

3.4.2.4 *Staphylococcus aureus* ATCC 25923

All bacterial strains were provided by the Pharmaceutical Science Laboratory, Faculty of Pharmaceutical Science, Ubon Ratchathani University, Thailand.

3.5 Equipment

3.5.1 Autoclave, Kokusan Enshinkimod H-88LL, Tomy Seiko Co., LTD. Japan

3.5.2 Balance, AL104, Mettler Toledo, Thailand

3.5.3 Centrifuge, Labofug 200 S/N 2500 – 10 Hanau Instruments, Germany

3.5.4 Colony plate counter, Sturt, Scientific industries, INC BO HEMIA, USA

3.5.5 Freeze dry, ScanVac, Scientific promotion Co., LTD, Thailand

3.5.6 Hot air oven, SUT6060, Heraeus instruments, Germany

3.5.7 Hot plate and stirrer, Torrey Pines Scientific, Gibthai Co. LTD, Thailand

3.5.8 Incubator, Memmert, Becthai Bangkok Equipment, and Chemical Co, LTD, Thailand

3.5.9 Laminar air flow, Holten Mod. HBB 2460 Scientific promotion. Co, LTD, USA

3.5.10 Microscope, Nikon/Alphaphot 2, Toledo, Switzerland

3.5.11 pH meter, Mettler, Toledo, Switzerland

3.5.12 Spectrophotometer, Novaspec II, Pharmacia LKB Biochrom, England

3.5.13 Vortex mixer, Thermolyne, Scientific industries, INC BO HEMIA, USA

3.5.14 Water bath, WNB29 with a flat cover, Memmert, Thailand

3.6 Methods

3.6.1 Sampling procedure of fermented foods

Fifty fermented food samples were collected from several local markets and supermarkets in Ubon Ratchathani and nearby provinces. Food samples such as, pickled fish, pickled kimchi, pickled crab, fermented minced fish, Nam and sausage (Appendix C) were conveyed directly to the laboratory and kept at 4^{o}C for further analysis.

3.6.2 Isolation of LAB

Ten grams of each sample were aseptically transferred to the sterile tube and 50 ml of saline (NaCl 0.85% w/v) were then added. The preparation was blended for

five minutes, and 100 µL of the samples were spread on MRS agar and incubated at 37ºC for 24 hours. The colonies of the LAB were randomly selected from the MRS agar plates of each sample with the morphology of a white color and convex shape. Then it transferred as a single colony on MRS agar plates by streak plate method and incubated at 37ºC for 24 hours. Each of the LAB samples were kept at 4ºC for further analysis and maintained in MRS broth containing 20% v/v sterile glycerol and stored at -80ºC.

3.6.3 Preliminary characterization of LAB by gram stain and catalase test

A single colony of LAB was heat-fixed with smears on the slide and stained with crystal violet for one minute, washed in tap water, covered with Gram's iodine for one minute, rewashed, decolorized for a few seconds in ethyl alcohol 95%, and counterstained for 30 seconds with Safranin O. The smears were washed thoroughly and gently air-dried then observed under the oil immersion objective lens. Gram-positive and rod or circular shaped bacteria were selected (Lim and Im, 2009).

A loopful of the LAB was placed on a slide, and few drops of 3% (v/v) hydrogen peroxide were added. The slides were observed for effervescence. Catalase-negative bacteria were selected, and *Staphylococcus aureus* ATCC 25923 were used as a positive control (Song *et al.*, 2010).

3.6.4 Screening of antibacterial activity of LAB isolates by cylinder-plate method

The screening of LAB isolates for antibacterial activity against pathogenic bacteria was determined by a cylinder-plate method. Six pathogenic bacteria comprising four Gram negative bacteria (*Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* DMST 4739, *Salmonella enteritidis* DMST 15676, *Salmonella typhimurium* DMST 560) and two Gram positive bacteria (*Bacillus cereus* DMST 5040 and *Staphylococcus aureus* ATCC 25923) were evaluated. The overnight culture of LAB were centrifuged at 8,000 rpm for 15 min at 4°C, and the supernatant was filter sterilized. The pathogenic bacteria (10^8 CFU/ml) were spread on MHA agar by a cotton swab. Then, the wells were made using a clean and sterile cylinder cup, and 180 µL of cell free culture supernatant (CFCS) was added into the cup. The plates were incubated at 37°C overnight. Finally, the diameter of the zone of inhibition was measured in millimeters (mm) using a Vernier

caliper. Oxytetracycline 50 µg/mL was used as the positive control, and MRS broth was used as the negative control.

3.6.5 Acid and bile salt tolerance

The LAB isolates that displayed the highest inhibition zone against the pathogenic bacteria were selected to assess the ability of the acid and bile salt tolerance. LAB were incubated in MRS broth at 37°C for 24 hours. Bacterial cells from the overnight cultures were centrifuged with 4,000 rpm, 10 min, 4°C, then the cell pellets were washed with PBS suspended in MRS broth to obtain the cell concentration approximately1.5 x 10^8 CFU/mL. One mL of LAB culture was added into 9 mL of PBS that was adjusted the pH value to pH 2, 3 and 6.5 using 1 M HCl and 3 M NaOH.

The viable cell counts were determined after incubation at 37°C for 0 and 4 hours by standard plate count on MRS agar. The ability of the isolates to grow in the presence of bile was determined by adding bacterial suspensions to MRS broth supplemented with 0.15 and 0.30% (w/v) bile salt. The viable cell counts were determined after incubation at 37°C for 0 and 4 hours, reflecting the time spent by food in the small intestine. The survivability of the LAB isolates under acidic and bile salt conditions was calculated using the following equation:

% Survival = [log no. of viable cell survived (CFU/ml)/log no. of initial viable cell (CFU/ml)] x 100

3.6.6 Antibacterial activity of the neutralized CFCS from LAB isolates

Three of the LAB strains (P09, P10 and P11) passed the acid and bile salt tolerance, were selected to determine the antibacterial activity after neutralization of CFCS for investigation the extracellular substance. The overnight culture of LAB isolates was centrifuged at 8,000 rpm for 15 min at 4°C, and the supernatant was collected and adjusted the pH to 6.5 with 3 N NaOH and were filter sterilized through a 0.22 mm-pore size membrane. The antibacterial activities of non-neutralized and neutralized CFCS were tested by cylinder plate method as described in section 3.6.4.

3.6.7 Safety test

From the characterization of selected potential probiotic strain, the strains of LAB, which had potential probiotic properties were selected to be determined for the safety test.

3.6.7.1 Antibiotic susceptibility test

LAB were grown in MRS broth at 37°C for 24 hours then they were adjusted to be 10^8 CFU/ml. Then, bacterial cells were spread onto the MRS agar plate using sterile cotton swab. The antibiotic discs used for the antibiotic susceptibility assay were sulphamethoxazole 25 µg, norfloxacin 10 µg, tetracycline 30 µg, ampicillin 10 µg, and gentamicin 10 µg and placed on agar and incubated at 37°C for 24 hours. The antibiotic susceptibility was reported with sensitivity, intermediate susceptibility, and resistance. The result was observed by measuring the diameter of the growth inhibition as a clear zone around each antibiotic disc (Kastner et al., 2006).

3.6.7.2 Hemolytic activity test

Hemolytic activity was determined by inoculating the strains on the Columbia Blood agar plates. After 48 hours of incubation at 37°C, the hemolytic reaction was recorded by observing the clear zone of the hydrolysis around the colonies (β-hemolysis), partial hydrolysis and the greenish zone (α-hemolysis), or no reaction (γ-hemolysis). *Staphylococcus aureus* ATCC 25923 was used as the positive control (Pisano et al., 2014).

3.6.7.3 DNase activity test

DNase test agar was used for detecting the deoxyribonuclease (DNase) activity of the bacteria. The DNA was hydrolyzed into oligonucleotides by the action of DNase nucleotides soluble in acid (after addition of 1 N HCl). LAB were grown in MRS broth at 37°C for 24 hours then they were adjusted to be 10^8 CFU/ml. The LAB culture was dropped on the DNase agar. After incubation at 37°C for 24 hours, the agar plate was flooded with 1 N HCl, and the clear zone around the growth was recorded. *Staphylococcus epidermidis* DMST 15505 was used for the negative control, and *Staphylococcus aureus* ATCC 25923 was used for the positive control (Singh et al., 2012).

3.6.8 Biochemical characterization and identification of the selected LAB

The isolates obtains exhibiting high safety properties were further assessed by API 50 CHL (BioMérieux, France) assay. LAB were cultivated in 20 ml of MRS broth and incubated at 30°C overnight, after which the culture was washed and resuspended into a medium of API 50 CHL. The turbidity of the suspension was determined by the McFarland method according to the instructions provided by the manufacturer. The cell suspension was applied to the API 50 CHL strip wells, and paraffin oil was pipetted into each well to create anaerobic conditions. The strips were incubated at incubation 30°C. The results were recorded after 24 hours and verified after 48 hours. The fermentation of the carbohydrates in the carbohydrate medium was indicated by a yellow color except for esculine (dark brown). The color reactions were scored against a chart provided by the manufacturer. The results were analyzed with API WEB (Bio-Merieux), and *Lactobacillus casei* TISTR 1341 was used as the control strain (Iranmanesh et al., 2016).

3.6.9 The 16S rDNA gene sequences of the selected LAB

The selected LAB were identified by 16S rDNA sequencing (Mahidol University-Osaka University Collaborative Research Center for Bioscience and Biotechnology, Mahidol University). The results were analyzed by the GenBank databases of the BLAST program of the National Center for Biotechnological Information (NCBI), USA (BLAST, 2019: Website).

3.6.10 Adhesion to the Caco-2 cell line

The selected potential probiotic strains was determined for the adhesion capacity to the human colon carcinoma cell line (Caco-2 cell). Standard *Lactobacillus casei* TISTR 1341 and *Lactobacillus plantarum* TISTR 541 was obtained from the Faculty of Pharmaceutical Science, Ubon Ratchathani University. Caco-2 cells were grown in a cell culture flask using a DMEM medium, 10% heat-inactivated fetal bovine serum, 100 ml streptomycin, 1% non-essential amino acid, and 100 IU/ml penicillin. Caco-2 cells were subsequently added into 24-well culture plates at a concentration of $2.5x10^5$ cells per well and allowed to differentiate for three days, while the medium was changed daily. The Caco-2 cells were incubated at 37°C in a 5% CO_2 atmosphere. Overnight cultures of the LAB isolates were centrifuged, washed twice with PBS, and resuspended in the same buffer. After that, the bacterial cell suspensions of LAB isolates

were added to each well, and the plates were incubated at 37ºC for four hours. After incubation, the wells were washed 3 times with PBS to remove non-adherent bacterial cells and then the Caco-2 cell were lysed with 0.1% Triton X-100 solution. The mixtures of lysed Caco-2 cells and LAB isolates were serially diluted and spread on the MRS agar plates to determine the number of viable adhered LAB isolates. The agar plates were incubated at 37ºC for 24-48 hours. The percentage of the bacterial adhesion on Caco-2 cells was calculated as a percentage of the viable bacteria according to their initial population (Rajoka et al., 2018).

3.6.11 Co-culture of probiotic and bacterial pathogens

The LAB strain showing the highest adhesion ability to the Caco-2 cell were chosen to further investigate the antibacterial activity by co-culture method. The co-culture assay were performed with 4 bacterial pathogens including *B. cereus* DMST 5040, *S. aureus* ATCC 25923, *E. coli* ATCC 25922 and *S. typhimurium* DMST 560. The LAB strain and the pathogenic strains were each incubated in the specific medium at 37°C for 24 hours (Kumar, Kundu and Debnath, 2018). The cells were removed from the culture broth and washed twice by using 30 ml of 0.85% (w/v) NaCl and centrifuged at 8,000 rpm for 15 min at 4°C. The LAB cells suspension was determined using a spectrophotometer at 625 nm, and the cell density was adjusted to be at $OD_{625} = 1.0$ and the cell suspensions of each of the pathogenic bacteria at 10^8 CFU/ml were the co-culture in the modified media with 1:1 MRS and TSB broth. All groups were cultivated in an erlenmeyer flask containing 100 ml of broth incubated at 37°C. The growth was monitored by plating onto selective agars at specific time points (0, 6, 12, 18, and 24 hours) over a period of 24 hours. The samples of 1 mL were taken and serially diluted in normal saline. A 100 µL aliquot was plated onto MRS agar for the probiotic strain, Phenol Red Egg Yolk Polymyxin (MYP) Agar Base for *B. cereus* DMST 5040, Mannitol Salt Agar (MSA) for *S. aureus* ATCC 25923, MacConkey Agar for *E. coli* ATCC 25922, and *Salmonella Shigella* (SS) Agar for *S. typhimurium* DMST 560. As the controls, 100 µL of each pathogen was used to be inoculated and kept under the same condition. All plates were incubated at 37°C for 48 hours for the MRS and selective agars. One mL was taken from each sample at each time point for the pH measurement (adapted from Likotrafiti (Likotrafiti et al., 2015).

3.6.12 Preparation of the synbiotic powder by the freeze-drying method

Freeze-drying of the probiotic strain and inulin powder (prebiotic) was performed. The LAB strain was grown in MRS broth at 37°C for 24 hours, centrifuged at 3500 rpm for 15 min at 4°C, and resuspended with normal saline (1.5 x 10^8 CFU/mL). It was supplemented with 3% (w/v) of inulin powder and then mixed with 10% (w/v) of skim milk or maltodextrin for the cryoprotective agents. The suspensions were transferred into the lyophilized flask under the aseptic conditions and frozen at -40°C for 24 hours. A freeze-drying procedure was operated at 0.1 mbar at -110°C for 18 hours (Dhewa, Pant and Mishra, 2014).

3.6.13 Survival of the synbiotic powder under simulated GI tract conditions

The survivability of synbiotic powder in a simulated gastrointestinal tract (GIT) was determined according to Maresca et al. (2018) with some modifications. The evaluation of the resistance to the simulated GIT conditions for the survivability of the synbiotic powder during storage at refrigerator temperature and room temperature was conducted for 24 hours. One gram of freeze-dried synbiotic powder was immediately suspended in 9 ml of saline (NaCl 0.85% w/v) and manually homogenized under aseptic conditions. One ml of culture from each tube was taken to be incubated in MRS broth at 37°C for 24 hours. Probiotic (5 mL, 2.5x10^9 CFU/mL) overnight culture was recovered by centrifugation at 3,500 rpm at 4°C for 10 min, washed twice with sterile saline, and suspended in simulated GI solutions.

For the mouth simulation, the cell pellet of the sample was resuspended with 2 mL of simulated saliva solution (1 L saliva solution contained 2.38 g Na_2HPO_4, 0.19 g KH_2PO_4, 8.0 g NaCl and 0.91 g α-amylase, adjusted to pH 6.75 with a phosphate buffer) and incubated for 10 min in a water bath at 37°C then centrifuged at 3,500 rpm at 4°C for 10 min. Aliquots were removed for counting viable cells (Li et al., 2019).

For the stomach simulation, the cell pellets of the sample were resuspended with 10 mL of simulated gastric solution (1 L gastric solution contained 3 g pepsin and 6.2 g NaCl, 0.22 g $CaCl_2$, 2.2 g KCl, and 1.2 g $NaHCO_3$ adjusted the pH to 2.5) in a shaking water bath (200 rpm) at 37°C and then centrifuged at 3,500 rpm at 4°C for 10 min. At 0, 30 and 60 min, the aliquots were taken to determine the number of viable cells.

For the intestinal simulation, the cell pellets of the sample were resuspended with 10 mL of simulated intestinal solution (1 L intestinal juice contained 0.1% pancreatin, 3 g bile salts, 1.2 g NaCl, 0.239 g KCl, and 6.4 g $NaHCO_3$ adjusted the pH to 7.0) in a shaking water bath (200 rpm) at 37°C and then centrifuged at 3,500 rpm at 4°C for 10 min. At 0, 2 and 4 hours, aliquots were taken to determine the number of viable cells (Maresca, Zotta and Mauriello, 2018).

For the control, using saline (NaCl 0.85% w/v) and 100 µL of each condition, aliquots were removed for counting the viable bacteria for the 10-fold serial dilution and spread on MRS agar at 37°C for 24-48 hours. The survival rate was measured.

3.6.14 Stability of the freeze dried synbiotic powder

A stability test was conducted under two storage conditions of the synbiotic powder at refrigerated temperature (4-8 °C) and room temperature (25-30 °C) all packages were stored in zip foil bags using aseptic technique. During this period, the viable cell was enumerated. Samples were taken every four weeks until 12 weeks of storage.The viability of the probiotic strain in the synbiotic powder was determined and expressed as CFU/mL (Dhewa, Pant and Mishra, 2014).

3.6.15 Anti-foodborne bacteria of the synbiotic powder

The synbiotic powder was stored at refrigerated temperature and room temperature, and the powder was collected for examining the anti-pathogenic bacteria at weeks 0, 1, 2, 3, 4, 8, and, 12. The antimicrobial activity of the synbiotic powder against the four pathogenic bacteria was determined by a cylinder-plate method. The pathogenic bacteria (*Escherichia coli* ATCC 25922, *Salmonella typhimurium* DMST 560, *Staphylococcus aureus* ATCC 25923 and *Bacillus cereus* DMST 5040) were evaluated for their potential antimicrobial activity. The powder was dissolved in normal saline and added into MRS broth for overnight culture by being placed in a shaking incubator at 37°C. Following this, it was centrifuged at 3,500 rpm/min for 15 min at 4°C, and the supernatant was filter sterilized. The pathogenic bacteria (10^7 CFU/ml) were spread on MHA agar by a cotton swab. The wells were made using a clean and sterile cylinder cup, and 180 µL of CFCS was added inside the cup. The plates were incubated at 37°C overnight.

Finally, the diameter of the zone of the inhibition was measured by using a Vernier caliper. Tetracycline 50 µg/ml was used as the positive control, and MRS broth was used as a negative control. (Beristain-Bauza et al., 2016).

3.7 Statistical analysis

Values are present as mean values and standard deviations of triplicate experiments. Independent t-test was used to test the significant difference between the viable count of each pathogen for the results obtained in the co-culture assay. Significant ANOVA results were tested with Tukey's Multiple Comparison Test in all assays and differences were considered statically significant if $p < 0.05$.

CHAPTER 4
RESULTS

4.1 Isolation and Characterization of Lactic Acid Bacteria (LAB) from Fermented Foods

LAB were isolated from 50 fermented foods; such as, pickled fish, pickled kimchi, pickled crab, fermented minced fish, and sausage. All the fermented foods were collected from several local markets and supermarkets in Ubon Ratchathani province and nearby provinces. In the isolation process of the LAB, the colonies on MRS agar were circular, creamy, smooth with an entire margin, and had growth on MRS Agar (Figure 4.1). 83 LAB were selected from the morphology comprising 68 rod-shaped isolates and 15 cocci-shaped cell isolates. LAB showing Gram positive and catalase test negative were selected for further characterization.

Figure 4.1 Colony morphology of the isolated LAB on MRS agar plate

4.2 Screening of LAB for probiotic properties

The obtained 83 LAB isolates from fermented foods were screened for antibacterial activity against six foodborne pathogens consisting of four Gram negative bacteria; *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* DMST 4739, *Salmonella enteritidis* DMST 15676, *Salmonella typhimurium* DMST 560, and two Gram positive bacteria; *Bacillus cereus* DMST 5040, and *Staphylococcus aureus* ATCC 25923. According to the results of antibacterial screening, 10 LAB isolates were chosen for acid and bile salt tolerance test.

4.2.1 Screening for the antibacterial activity of LAB

83 LAB isolates were tested for antibacterial activity against six pathogenic bacteria. The results are shown in Table 4.1.

Table 4.1 Antibacterial activity of 83 LAB isolates

No.	Sample	Antibacterial activity					
		Gram negative bacteria				Gram positive bacteria	
		E. coli	*P. aeruginosa*	*S. enteritidis*	*S. typhimurium*	*B. cereus*	*S. aureus*
1	KJ 1	+	++	+	+	+	++
2	KJ 2	++	+	+	+	+	++
3	KJ 3	+	+	+	+	+	++
4	KJ 5	++	++	++	+	+	++
5	FC 2	+	++	+	+	-	+++
6	FFC 1	-	-	+	-	-	-
7	FFC 2	+	+	+	-	+	+
8	FFC 5	+	++	+	+	+	++
9	FFC 6	+	+	+	-	+	+
10	FFC 7	+	-	+	-	-	+
11	FFPR 1	+	+	+	+	+	++
12	FFPR 2	+	++	+	++	+	++++
13	FFPR 3	+	+	+	+	+	++
14	FFPR 4	+	+	+	+	+	++
15	FFPR 5	+	+	+	+	+	+
16	FFPR 6	+	+	+	-	+	++
17	FFPR 7	+	+	+	-	+	+
18	FFPR 8	+	++	+	+	+	+
19	FFPS 1	+	++	++	+	-	++++
20	FFPS 2	+	++	+	+	-	+++
21	FFPS 3	+	++	+	+	-	+++
22	FFPS 4	-	-	+	-	-	-
23	FFPS 5	+	+	+	+	+	+

Table 4.1 Antibacterial activity of 83 LAB isolates (Continued)

No.	Sample	Antibacterial activity					
		Gram negative bacteria				Gram positive bacteria	
		E. coli	*P. aeruginosa*	*S. enteritidis*	*S. typhimurium*	*B. cereus*	*S. aureus*
24	FFPS 6	+	+	+	-	+	++
25	MR 1	+	++	+	+	-	++
26	MR 2	+	+	-	++	-	+
27	MR 3	+	+	+	+	+	++
28	MR 4	+	+	+	+	+	+
29	MR 5	-	-	-	-	-	-
30	PSS 1	+	+	+	++	+	+
31	PSS 2	+	+	+	+	+	+
32	PSS 3	+	+	+	+	+	+
33	PSS 5	+	+	+	-	+	+
34	P01	++	+++	+++	++	++	++
35	P02	++	+++	+++	+	++	++
36	P03	++	++	++	+++	++	++
37	P04	++	+++	+++	+	++	++
38	P05	++	++	++	+	+++	++
39	P06	++	++	+++	+	++	++++
40	P07	++	++	+++	+	++	++
41	P08	++	++	++	+	++	++
42	P09	++	++	+++	+	++	++
43	P10	+++	++	+++	++	++	++
44	P11	++	+++	+++	+	+++	+
45	P13	+	+	+	+	+	+
46	P14	+	++	+	+	+	+

Table 4.1 Antibacterial activity of 83 LAB isolates (Continued)

No.	Sample	Antibacterial activity					
		Gram negative bacteria				Gram positive bacteria	
		E. coli	*P. aeruginosa*	*S. enteritidis*	*S. typhimurium*	*B. cereus*	*S. aureus*
47	P15	+	+	+	+	+	+
48	P19	+	+	+	++	+	+
49	P20	+	+	+	+	+	+
50	P21	+	+	+	+	+	+
51	P22	+	+	+	+	+	+
52	P23	+	+	+	+	+	+
53	P25	+	++	+	+	+	+
54	P26	+	+	+	+	+	+
55	P27	+	+	+	+	+	+
56	P28	+	+	+	+	+	-
57	P29	+	+	+	+	+	+
58	P30	+	+	+	+	+	+
59	P33	+	+	+	+	+	+
60	P34	+	+	+	+	+	+
61	P35	+	++	+	+	+	+
62	P56	+	++	+	+	+	++
63	P57	+	+	+	+	+	++
64	P59	+	+	+	+	+	++
65	P61	+	-	+	+	+	++
66	P62	+	-	+	+	+	++
67	P63	+	-	+	+	+	++
68	P64	+	-	+	+	+	++
69	P65	+	-	+	+	+	++

Table 4.1 Antibacterial activity of 83 LAB isolates (Continued)

No.	Sample	Antibacterial activity					
		Gram negative bacteria				Gram positive bacteria	
		E. coli	*P. aeruginosa*	*S. enteritidis*	*S. typhimurium*	*B. cereus*	*S. aureus*
70	P67	+	++	+	+	+	++
71	P69	+	++	+	+	+	++
72	P70	+	++	+	+	+	++
73	P71	+	+	+	+	+	+++
74	P72	+	+	+	+	+	++
75	P73	+	-	+	+	+	++
76	P74	+	-	+	+	+	++
77	P75	+	+	+	+	+	++
78	P76	-	-	+	-	+	-
79	P77	-	+	+	-	-	+
80	P78	-	+	+	-	-	+
81	P79	-	-	-	+	-	-
82	P80	+	+	-	+	-	+
83	P81	-	+	-	-	-	+
84	MRS broth	-	-	-	-	-	-
85	Oxytetracycline 50 µg/ml	+++	++++	+++	++++	++++	++++

Note: (++++): ≥ 20.00 mm inhibition zone, (+++): 16.00-20.00 mm inhibition zone, (++): 12.00-16.00 mm inhibition zone, (+): 8.00-12.00 mm inhibition zone and (-): no inhibition zone

Table 4.2 Antibacterial activity of 10 selected LAB isolates

No.	LAB Isolates	Inhibition zone (mm.)					
		Gram negative bacteria				Gram positive bacteria	
		E. coli	*P. aeruginosa*	*S. enteritidis*	*S. typhimurium*	*B. cereus*	*S. aureus*
1	FFPR 2	11.67±0.58	15.00±1.41	12.00±1.00	15.00±1.41	10.67±0.58	21.00±1.41
2	FFPS 1	11.33±1.53	14.67±1.53	12.67±0.58	12.00±1.73	-	21.00±1.00
3	P01	16.33±1.53	18.00±1.00	17.00±1.41	15.50±0.71	14.00±1.73	13.33±1.53
4	P02	14.67±1.15	18.00±1.00	17.33±0.58	11.00±0.00	16.00±1.41	15.67±1.15
5	P03	15.67±0.58	15.50±0.71	16.33±1.15	17.33±0.58	13.00±1.00	13.00±1.00
6	P05	15.67±0.58	14.00±0.00	16.00±1.41	11.00±0.00	18.00±1.00	14.00±1.73
7	P06	16.33±1.15	15.33±0.58	16.67±1.53	11.33±1.53	12.50±0.71	21.00±1.00
8	P09	16.33±0.58	16.33±1.53	19.00±1.00	9.67±0.58	14.50±0.71	13.00±0.00
9	P10	18.00±1.00	16.00±1.00	19.00±1.00	15.00±0.00	15.50±0.71	14.50±0.71
10	P11	16.33±0.58	17.00±1.41	16.50±2.12	11.50±0.71	18.00±1.00	11.67±0.58
11	Oxytetracycline 50 µg/ml	19.56±1.17	21.33±1.53	17.44±2.17	28.56±1.71	33.56±2.22	31.33±1.53

Note: The results showed as a mean ± SD of a triplicated experiments. (-): no inhibition zone

According to the results of the antibacterial activities, 10 LAB isolates displayed the highest inhibition zone against each of the pathogenic bacteria were selected for further characterization. The inhibition zone of FFPR2 and FFPS1 against *S. aureus* was 21.00±1.41 mm., and 21.00±1.00 mm, respectively. The isolate P06 also showed the high inhibition zone against *S. aureus* at 21.00±1.00 mm. The isolates P01 and P02 exhibited the highest inhibition zone against *P. aeruginosa* (18.00±1.00 mm). P03 showed the inhibition zone against *S. typhimurium* at 17.33±0.58 mm. P05 and P11 could inhibit the *B. cereus* at18.00±1.00 mm., The isolates P09 and P10 showed the same inhibition zone against *S. enteritidis* at 19.00±1.00 mm. And the isolates P10 exhibited the highest inhibitory activity against *E. coli* at the inhibition zone of 18.00±1.00 mm. Therefore, 10 LAB isolates (FFPR2, FFPS1, P01, P02, P03, P05, P06, P09, P10, and P11 isolates) were then selected for the next experiments as they showed the highest clear zone against each pathogenic strain. Moreover, they exhibited antibacterial activity against all six pathogenic bacteria except the isolate FFPS1 that did not inhibit *B. cereus.* Table 4.2 shows the inhibition zone of those 10 LAB isolates.

4.2.2 Acid and bile salt tolerance

To consider the bacteria to be a potential probiotic, it should possess several desirable characteristics like overcoming a low pH environment in the gastrointestinal (GI) tract and arriving at the site of action in a viable physiological state. For this consideration, the tested strains presented a low pH resistance, and the survival rate of the isolates was observed. According to the antibacterial screening activity, 10 LAB isolates were selected to determine the acid and bile salt tolerance. All of the isolates showed a survival rate of more than 90% (10^6–10^8 CFU/mL) (Figure 4.2). The P02, P05, P06, P09, P10, P11 showed a high survival rate more than 100% (10^6–10^7 CFU/mL) at pH 3, and, the P09, P10, and P11 also presented a high survival rate more than 100% (10^6–10^7 CFU/mL) at pH 2. From the report of health benefits of probiotics showed that the minimum effective dose of probiotics should have 10^6–10^9 CFU/mL) in the product (Kechagia, 2013), thus in this experiment represented that the selected LAB could be used as a potential probiotic.

Table 4.3 Acid tolerance of 10 selected LAB isolates

Isolates	pH value	Log CFU/mL (mean ± SD)		% Survival
		0 h	3 h	
FFPR 2	pH 6.5	6.87±0.01	6.80±0.19	98.98
	pH 3	6.99±0.03	6.94±0.01	99.29
	pH 2	6.82±0.21	6.13±0.69	90.06
FFPS 1	pH 6.5	7.61±0.08	7.86±0.05	103.32
	pH 3	7.21±0.02	7.03±0.57	97.53
	pH 2	6.95±0.19	6.57±0.08	94.56
P01	pH 6.5	6.76±0.01	6.98±0.13	103.29
	pH 3	6.67±0.23	6.42±0.18	96.22
	pH 2	7.06±0.06	6.97±0.02	98.68
P02	pH 6.5	7.64±0.12	8.11±0.05	106.10
	pH 3	7.61±0.25	7.84±0.08	103.17
	pH 2	7.25±0.10	7.18±0.01	99.07
P03	pH 6.5	6.88±0.00	6.91±0.10	100.44
	pH 3	6.86±0.14	6.82±0.13	99.45
	pH 2	6.78±0.01	6.68±0.17	98.62
P05	pH 6.5	6.67±0.03	6.93±0.21	103.83
	pH 3	6.69±0.13	6.75±0.14	101.06
	pH 2	6.30±0.19	6.21±0.01	98.60
P06	pH 6.5	6.42±0.04	7.26±0.33	113.07
	pH 3	6.43±0.07	6.70±0.01	104.31
	pH 2	6.33±0.07	6.30±0.19	99.54
P09	pH 6.5	6.74±0.10	7.03±0.13	104.32
	pH 3	6.65±0.09	6.89±0.05	103.75
	pH 2	6.51±0.03	6.74±0.08	103.53
P10	pH 6.5	7.57±0.05	7.59±0.02	100.20
	pH 3	7.47±0.02	7.47±0.01	100.07
	pH 2	7.30±0.12	7.36±0.05	100.74
P11	pH 6.5	6.99±0.16	7.39±0.04	105.68
	pH 3	7.33±0.25	7.65±0.26	104.52
	pH 2	6.81±0.09	7.09±0.43	104.19

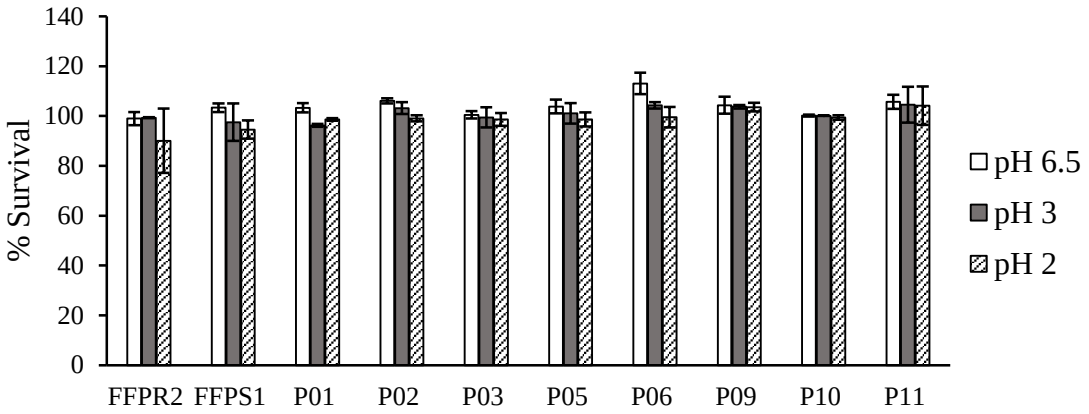


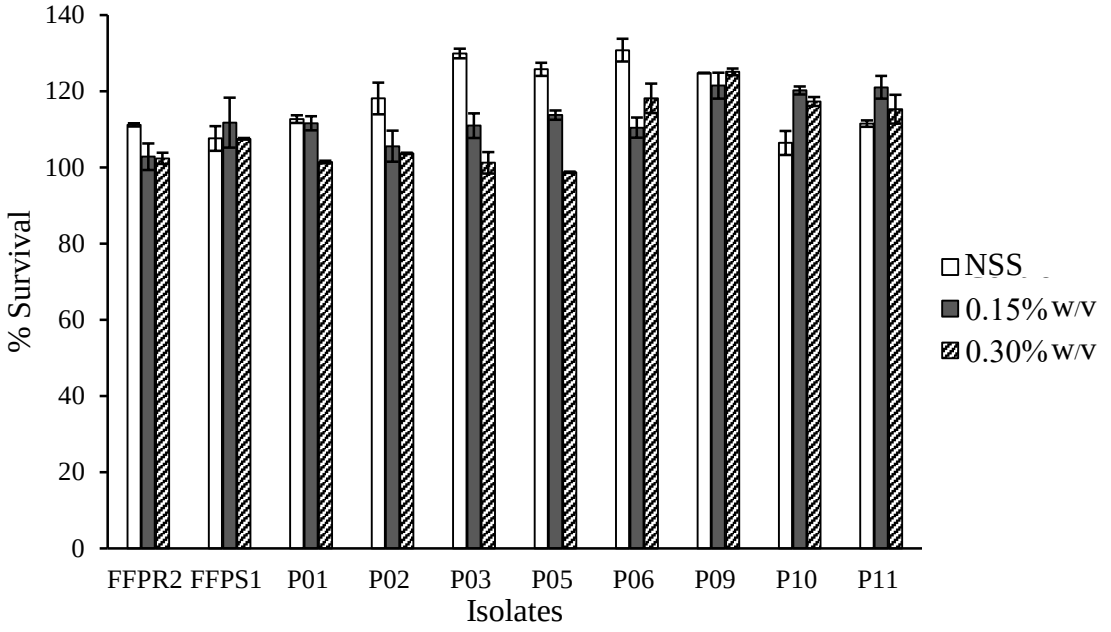
Figure 4.2 The survival rate of 10 LAB isolates under acidic conditions (pH 2 and pH 3) for 3 h. The results showed as a mean ± SD of a triplicated experiments

Bile plays a fundamental role in the specific and non-specific defense mechanism of the digestive system, and the magnitude of its inhibitory effect is determined primarily by the bile salt concentrations. Therefore, bile tolerance was considered as an important characteristic of the LAB strains, which enabled them to survive to grow and exert their action in the gastrointestinal transit.

The results indicated that at a 0.15% concentration of the bile salt, there was a slight effect to the selected LAB, especially, P09, P10, and P11 that showed a very high bile salt tolerance with a more than 120% (10^7–10^8 CFU/mL) survival rate. Additionally, P06, P09, P10, and P11 displayed a very high bile salt tolerance with a more than 110% (10^7–10^8 CFU/mL) survival rate at the 0.30% concentration of bile salt (Figure 4.3). The data showed minimum effective dose more than 10^6 CFU/mL probiotic should be consumed daily for the probiotic effect (Kechagia, 2013).

Table 4.4 Bile salt tolerance of 10 selected LAB isolates

Isolates	Bile salt concentration	Log CFU/mL (mean ± SD)		% Survival
		0 h	4 h	
FFPR 2	0.15 %	7.57±0.06	7.78±0.21	102.80
	0.3 %	7.22±0.09	7.39±0.01	102.35
	NSS	8.55±0.02	9.50±0.05	111.14
FFPS 1	0.15 %	6.94±0.60	7.73±0.22	111.72
	0.3 %	7.35±0.10	7.90±0.09	107.47
	NSS	7.61±0.05	8.19±0.30	107.59
P01	0.15 %	6.76±0.14	7.54±0.03	111.53
	0.3 %	6.64±0.03	6.73±0.01	101.41
	NSS	6.57±0.04	7.40±0.02	112.68
P02	0.15 %	7.24±0.24	7.63±0.05	105.55
	0.3 %	7.17±0.02	7.43±0.01	103.65
	NSS	7.44±0.01	8.78±0.32	118.09
P03	0.15 %	6.66±0.21	7.39±0.01	110.94
	0.3 %	6.52±0.01	6.60±0.19	101.20
	NSS	6.64±0.01	8.62±0.10	129.89
P05	0.15 %	6.73±0.29	7.65±0.25	113.70
	0.3 %	6.83±0.09	6.74±0.07	98.70
	NSS	7.01±0.10	8.82±0.01	125.73
P06	0.15 %	7.50±0.02	8.28±0.22	110.41
	0.3 %	6.90±0.02	8.15±0.29	118.10
	NSS	7.13±0.23	9.31±0.09	130.73
P09	0.15 %	6.69±0.05	8.13±0.29	121.45
	0.3 %	6.50±0.04	8.13±0.01	125.06
	NSS	6.71±0.00	8.37±0.01	124.76
P10	0.15 %	6.47±0.01	7.78±0.05	120.17
	0.3 %	6.72±0.01	7.88±0.07	117.27
	NSS	6.68±0.10	7.10±0.10	106.39
P11	0.15 %	6.93±0.13	8.39±0.05	121.00
	0.3 %	6.35±0.11	7.31±0.12	115.22
	NSS	6.66±0.07	7.43±0.13	111.49



The results showed as a mean ± SD of a triplicated experiments

Figure 4.3 The survival rate of 10 LAB isolates in MRS broth treated with 0.15% and 0.30% bile salt and without bile salt as a control group for 4 h.

4.3 Characterization of the selected potential probiotic strains

According to the previous experiments, the isolates P09, P10 and P11 showed antimicrobial against all of six bacterial pathogens and showed a high survival rate under acid and bile salt condition. The isolates P09, P10, and P11 were thus selected for characterization of the potential probiotic properties. In the following experiments, those three isolates were evaluated for their antibacterial activity after neutralization, safety test, strain identification, Caco-2 cell adherence ability, and co-culture assay.

4.3.1 Antimicrobial activity of the neutralized cell-free supernatant

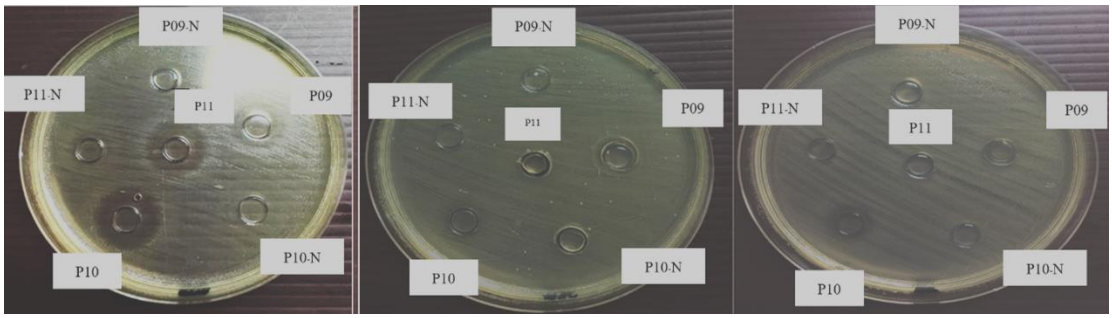
From the previous results of the acid and bile salt tolerance, P09, P10, and P11 were selected for the next experiments because they represented a high acid resistance at pH 2 and 3 and high bile salt resistance at 0.15% w/v and 0.30% w/v; therefore, the three isolates were selected to determine the antibacterial activity after neutralization of the cell-free supernatant to pH 6.5 for examining the LAB producing substance against the six pathogenic bacteria.

This assay neutralized the acid of the produced substance in the cell-free culture supernatant. There was no inhibition zone observed in any neutralized CFCS as shown in Table 4.5 and Figure 4.4. This result indicated that the antimicrobial activities of the isolates P09, P10, and P11 were likely involve in the organic acid production by the LAB strains.

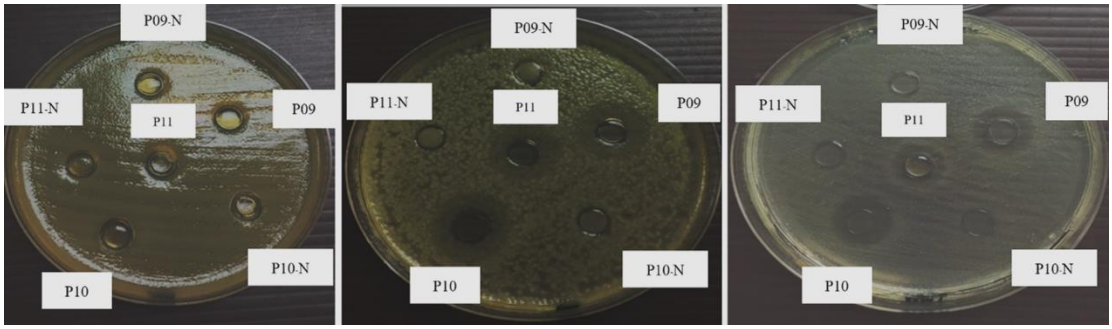
Table 4.5 Antimicrobial activity of cell-free culture supernatant (CFCS)

Samples	Antimicrobial activity					
	Gram negative bacteria				Gram positive bacteria	
	E. coli	***P. aeruginosa***	***S. enteritidis***	***S. typhimurium***	***B. cereus***	***S. aureus***
P09 Non-neutralized CFCS	++	++	+++	+	+	+++
P09 Neutralized CFCS	-	-	-	-	-	-
P10 Non-neutralized CFCS	+++	+++	+++	++	+	++
P10 Neutralized CFCS	-	-	-	-	-	-
P11 Non-neutralized CFCS	++	+++	++	+	+++	+
P11 Neutralized CFCS	-	-	-	-	-	-
MRS broth	-	-	-	-	-	-
Oxytetracycline 50 µg/mL	++++	++++	++++	++++	++++	++++

Note: (++++): ≥ 20.00 mm inhibition zone, (+++): 16.00-20.00 mm inhibition zone, (++): 12.00-16.00 mm inhibition zone, (+): 8.00-12.00 mm inhibition zone and (-): no inhibition zone



E. coli ***P. aeruginosa*** ***S. enteritidis***



S. typhimurium ***B. cereus*** ***S. aureus***

Note: P09-N; P09 Neutralized CFCS, P09; P09 Non-neutralized CFCS, P10-N; P10 Neutralized CFCS, P10; P10 Non-neutralized CFCS, P11-N; P11 Neutralized CFCS, P11; P11 Non-neutralized CFCS, respectively.

Figure 4.4 Inhibitory effects of P09, P10, and P11 against pathogenic bacteria

4.3.2 Safety test

4.3.2.1 Antibiotic susceptibility of the LAB

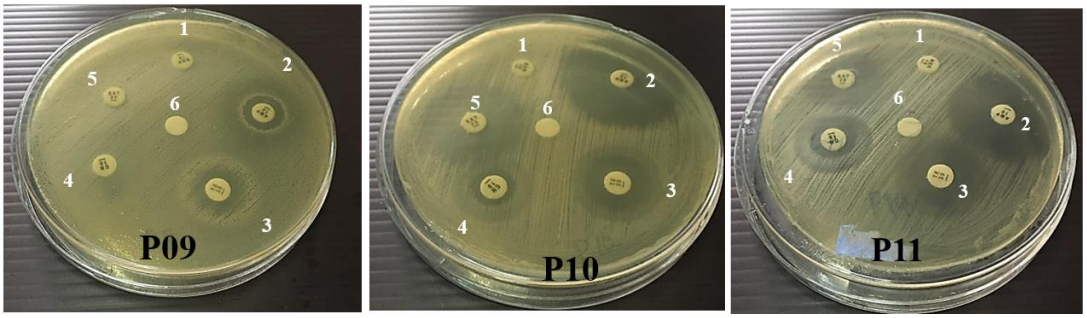
In this antibiotic susceptibility test, the selected LAB P09, P10, and P11 were tested for their susceptibility to five antibiotics by the disc diffusion method (Figure 4.5).

The three isolates (P09, P10, and P11) were interpreted to be resistant to norfloxacin, and only the P09 isolate was resistant to gentamicin and sulfamethoxazole. The isolates P09, P10 and P11 showed intermediate sensitivity, high sensitivity, and sensitivity to ampicillin, respectively. In contrast, all three isolates showed intermediate sensitivity to tetracycline. The isolate P09 a was also resistant to gentamicin and sulfamethoxazole. Whereas, the isolates P10 and P11 showed sensitivity and intermediate sensitivity towards sulfamethoxazole, respectively (Table 4.6).

Table 4.6 Antibiotic susceptibility profile of the selected LAB isolates

Isolates	Norfloxacin (10 µg)	Ampicillin (10 µg)	Tetracycline (30 µg)	Gentamicin (10 µg)	Sulphamethoxazole (25 µg)
P09	R	I	I	R	R
P10	R	H	I	I	S
P11	R	S	I	I	I

Note: Highly sensitive (H) >31.00 mm, Sensitive (S) 21.00-30.00 mm, Intermediate (I) 10.00-20.00 mm, Resistant (R) to antibiotics <10.00 mm



Note: No. 1-6 represent Norfloxacin 10 µg; Ampicillin 10 µg; Tetracycline 30 µg; Gentamicin 10 µg; Sulphamethoxazole 25 µg and control, respectively.

Figure 4.5 Antibiotic susceptibility of isolates P09, P10, and P11

4.3.2.2 Hemolytic activity

All three isolates were evaluated for hemolytic activity on sheep blood agar. The results showed that P9, P10, and P11 did not show any red blood cell digestion reactions (γ-hemolysis) compared with the control group. The positive control was *S. aureus* ATCC 25923, which displayed a red blood cell digestion zone around the colony (β-hemolysis) (Figure 4.6).

Hemolysis was a known virulence factor among the pathogenic microorganisms. The absence of hemolytic activity was considered as a safe prerequisite for the selection of probiotic strain.

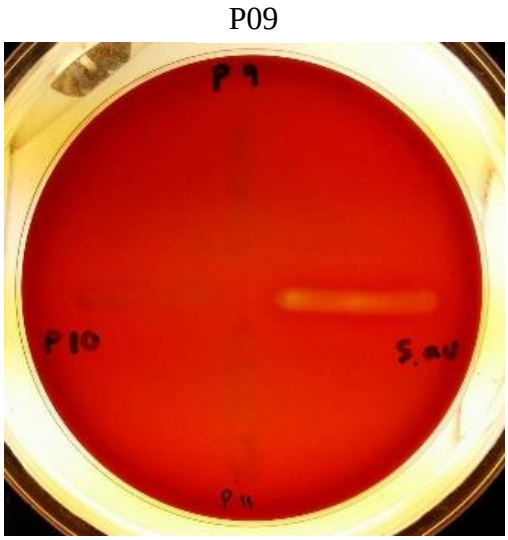
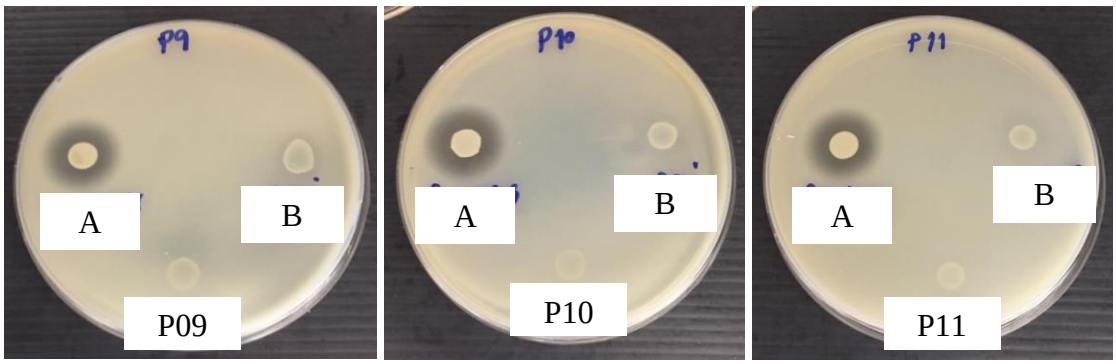


Figure 4.6 Haemolytic activity assay of the isolates P09, P10, and P11

4.3.2.3 DNase activity test

This method tested the deoxyribonuclease (DNase) activity by observing the digestive zone on DNase agar. The results showed that P9, P10 and P11 did not produce any DNase activity. The positive control, *S. aureus* ATCC 25923 showed clear zone around the colony and the negative control, *S. epidermidis* DMST 15505 did not show a clear zone around the colony (Figure 4.7). All of the isolates were considered as a safe prerequisite for the selection of the probiotic strain.



Note: Positive control (A); *S. aureus* ATCC 25923 and negative control (B); *S. epidermidis* DMST 15505

Figure 4.7 DNase activity test of the isolates P09, P10 and P11

4.3.3 Biochemical characterization test

An analysis of carbohydrate fermentation by the selected strains was done using the API 50 CHL system kit. All the selected LAB, including *L. casei* TISTR 1341, fermented the following carbohydrates ribose, D-glucose, D-fructose, N-acetly-glucosamine, Arbutine, Salicine, Maltose, Saccharose, and Trehalose.

Table 4.7 Carbohydrate fermentation pattern of LAB isolates at 37°C

No.	Carbohydrates	LAB isolates			
		P09	**P10**	**P11**	***L. casei***
0	Control	-	-	-	-
1	Glycerol	-	-	-	-
2	Erythritol	-	-	-	-
3	D-Arabinose	-	-	-	-
4	L-Arabinose	-	-	-	-
5	Ribose	+	+	+	+
6	D Xylose	-	-	-	-
7	L-Xylose	-	-	-	-
8	Adonitol	-	-	-	-
9	β-methyl-xyloside	-	-	-	-
10	Galactose	+	+	+	-
11	D-Glucose	+	+	+	+
12	D-Fructose	+	+	+	+
13	D-Mannose	+	+	+	-
14	L-Sorbose	-	-	-	-
15	Rhamnose	-	-	-	-
16	Dulcitol	-	-	-	-
17	Inositol	-	-	-	-
18	Mannitol	+	+	+	-
19	Sorbitol	-	+	+	-

Table 4.7 Carbohydrate fermentation pattern of LAB isolates at 37°C (continued)

No.	Carbohydrates	LAB isolates			
		P09	**P10**	**P11**	***L. casei***
20	α-Methyt-D-mannoside	+	-	-	-
21	α-Methyt-D-glucoside	-	-	-	-
22	N-Acetly-glucosamine	+	+	+	+
23	Amygdaline	+	+	+	-
24	Arbutine	+	+	+	+
25	Esculin	-	-	-	-
26	Salicine	+	+	+	+
27	Cellobiose	+	+	+	-
28	Maltose	+	+	+	+
29	Lactose	+	+	+	-
30	Melibiose	+	+	-	-
31	Saccharose	+	+	+	+
32	Trehalose	+	+	+	+
33	Inuline	-	-	-	-
34	Melezitose	+	+	-	-
35	D-Raffinose	-	-	-	-
36	Amidon	-	-	-	-
37	Glycogene	-	-	-	-
38	Xylitol	-	-	-	-
39	β-Gentiobiose	+	+	+	-
40	D-Turanose	-	-	-	-
41	D-Lyxose	-	-	-	-
42	D-Tagatose	-	-	-	-
43	D-Fucose	-	-	-	-
44	L-Fucose	-	-	-	-

Table 4.7 Carbohydrate fermentation pattern of LAB isolates at 37°C (continued)

No.	Carbohydrates	LAB isolates			
		P09	**P10**	**P11**	***L. casei***
45	D-Arabitol	-	-	-	-
46	L- Arabitol	-	-	-	-
47	Gluconate	+	+	-	-
48	2-keto gluconate	-	-	-	-
49	5-keto gluconate	-	-	-	-

Note: (+); acid production and (-); no acid produced

4.3.4 LAB identification by 16S rDNA gene analysis

The molecular identification of the selected LAB strains was investigated by 16S rDNA gene analysis. All three of the selected strains were identified as *Lactobacillus plantarum* (Table 4.8).

Table 4.8 The 16S rDNA gene sequences of LAB isolates

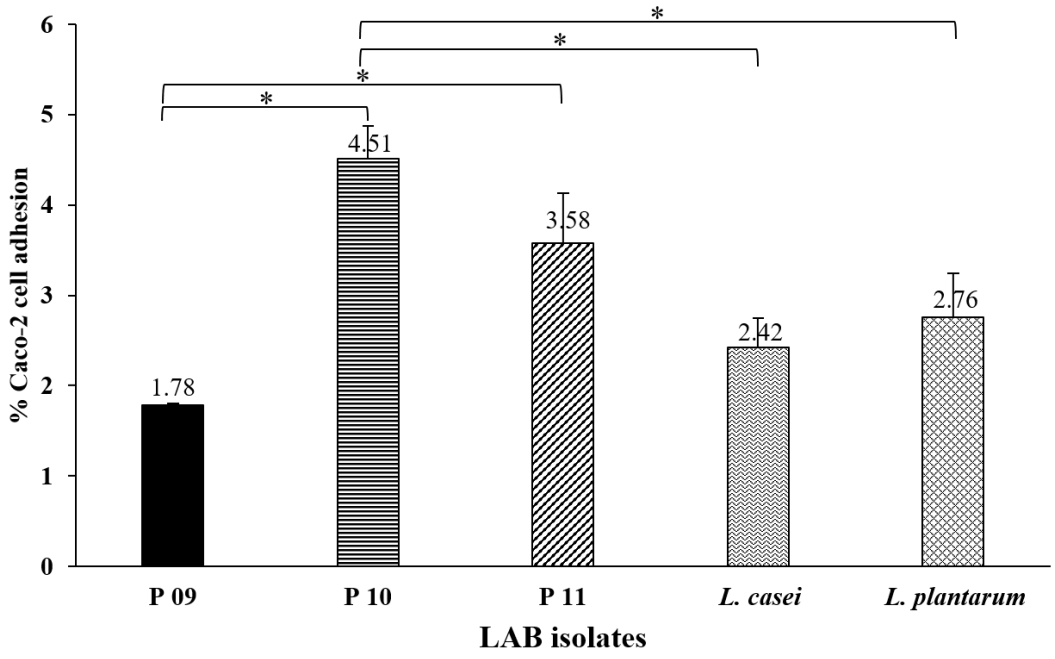
Isolates	Nucleotide sequences	% identity
P09	GCTGGCGGCGTGCCTAATACATGCAAGTCGAACGAACTCTGGTATTGATT GGTGCTTGCATCATGATTTACATTTGAGTGAGTGGCGAACTGGTGAGTAA CACGTGGGAAACCTGCCCAGAAGCGGGGGATAACACCTGGAAACAGAT GCTAATACCGCATAACAACTTGGACCGCATGGTCCGAGTTTGAAAGATG GCTTCGGCTATCACTTTTGGATGGTCCCGCGGCGTATTAGCTAGATGGTG GGGTAACGGCTCACCATGGCAATGATACGTAGCCGACCTGAGAGGGTAA TCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGC AGTAGGGAATCTTCCACAATGGACGAAAGTCTGATGGAGCAACGCCGCG TGAGTGAAGAAGGGTTTCGGCTCGTAAAACTCTGTTGTTAAAGAAGAAC ATATCTGAGAGTAACTGTTCAGGTATTGACGGTATTTAACCAGAAAGCC ACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGT TGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTTTTAAGTCTG ATGTGAAAGCCTTCGGCTCAACCGAAGAAGTGCATCGGAAACTGGGAAA CTTGAGTGCAGAAGAGGACAGTGGAACTCCATGTGTAGCGGTGAAATGC GTAGATATATGGAAGAACACCAGTGGCGAAGGCGGCTGTCTGGTCTGTA ACTGACGCTGAGGCTCGAAAGTATGGGTAGCAAACAGGATTAGATACCC TGGTAGTCCATACCGTAAACGATGAATGCTAAGTGTTGGAGGGTTTCCGC CCTTCAGTGCTGCAGCTAACGCATTAAGCATTCCGCCTGGGGAGTACGGC CGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTG GAGCATGTGGTTTAATTCGAAGCTACGCGAAGAACCTTACCAGGTCTTG ACATACTATGCAAATCTAAGAGATTAGACGTTCCCTTCGGGGACATGGA TACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTA AGTCCCGCAACGAGCGCAACCCTTATTATCAGTTGCCAGCATTAAGTTGG GCACTCTGGTGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGAC GTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGG ATGGTACAACGAGTTGCGAACTCGCGAGAGTAAGCTAATCTCTTAAAGC CATTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGTCGGAAT CGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTT GTACACACCGCCCGTCACACCATGAGAGTTTGTAACACCCAAAGTCGGT GGGGTAACCTTTTAGGAACCAGCCGCCTAAGGTGGGACAGATGATTAGG GTGAAGTCGTAACAAGGTAGCCGTAGGAGAACCTGCGGTTGGATCACC	94.79% *Lactobacillus plantarum* strain CIP 103151
P10	GCTGGCGGCGTGCCTAATACATGCAAGTCGAACGAACTCTGGTATTGATT GGTGCTTGCATCATGATTTACATTTGAGTGAGTGGCGAACTGGTGAGTAA CACGTGGGAAACCTGCCCAGAAGCGGGGGATAACACCTGGAAACAGAT GCTAATACCGCATAACAACTTGGACCGCATGGTCCGAGTTTGAAAGATG GCTTCGGCTATCACTTTTGGATGGTCCCGCGGCGTATTAGCTAGATGGTG GGGTAACGGCTCACCATGGCAATGATACGTAGCCGACCTGAGAGGGTAA TCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGC AGTAGGGAATCTTCCACAATGGACGAAAGTCTGATGGAGCAACGCCGCG TGAGTGAAGAAGGGTTTCGGCTCGTAAAACTCTGTTGTTAAAGAAGAAC ATATCTGAGAGTAACTGTTCAGGTATTGACGGTATTTAACCAGAAAGCC ACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGT TGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTTTTAAGTCTG ATGTGAAAGCCTTCGGCTCAACCGAAGAAGTGCATCGGAAACTGGGAAA CTTGAGTGCAGAAGAGGACAGTGGAACTCCATGTGTAGCGGTGAAATGC GTAGATATATGGAAGAACACCAGTGGCGAAGGCGGCTGTCTGGTCTGTA ACTGACGCTGAGGCTCGAAAGTATGGGTAGCAAACAGGATTAGATACCC TGGTAGTCCATACCGTAAACGATGAATGCTAAGTGTTGGAGGGTTTCCGC CCTTCAGTGCTGCAGCTAACGCATTAAGCATTCCGCCTGGGGAGTACGGC CGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTG GAGCATGTGGTTTAATTCGAAGCTACGCGAAGAACCTTACCAGGTCTTG ACATACTATGCAAATCTAAGAGATTAGACGTTCCCTTCGGGGACATGGA TACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTA AGTCCCGCAACGAGCGCAACCCTTATTATCAGTTGCCAGCATTAAGTTGG GCACTCTGGTGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGAC GTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGG ATGGTACAACGAGTTGCGAACTCGCGAGAGTAAGCTAATCTCTTAAAGC CATTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGTCGGAAT CGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTT GTACACACCGCCCGTCACACCATGAGAGTTTGTAACACCCAAAGTCGGT GGGGTAACCTTTTAGGAACCAGCCGCCTAAGGTGGGACAGATGATTAGG GTGAAGTCGTAACAAGGTAGCCGTAGGAGAACCTGCGGTTGGATCACC	95.52% *Lactobacillus plantarum* strain CIP 103151

Table 4.8 The 16S rDNA gene sequences of LAB isolates (continued)

Isolates	Nucleotide sequences	% identity
P11	GCTGGCGGCGTGCCTAATACATGCAAGTCGAACGAACTCTGGTATTGATTG GTGCTTGCATCATGATTTACATTTGAGTGAGTGGCGAACTGGTGAGTAACA CGTGGGAAACCTGCCCAGAAGCGGGGGATAACACCTGGAAACAGATGCTA ATACCGCATAACAACTTGGACCGCATGGTCCGAGTTTGAAAGATGGCTTCG GCTATCACTTTTGGATGGTCCCGCGGCGTATTAGCTAGATGGTGGGGTAAC GGCTCACCATGGCAATGATACGTAGCCGACCTGAGAGGGTAATCGGCCAC ATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAA TCTTCCACAATGGACGAAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGA AGGGTTTCGGCTCGTAAAACTCTGTTGTTAAAGAAGAACATATCTGAGAGT AACTGTTCAGGTATTGACGGTATTTAACCAGAAAGCCACGGCTAACTACGT GCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGG GCGTAAAGCGAGCGCAGGCGGTTTTTTAAGTCTGATGTGAAAGCCTTCGGC TCAACCGAAGAAGTGCATCGGAAACTGGGAAACTTGAGTGCAGAAGAGGA CAGTGGAACTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAAGAACA CCAGTGGCGAAGGCGGCTGTCTGGTCTGTAACTGACGCTGAGGCTCGAAA GTATGGGTAGCAAACAGGATTAGATACCCTGGTAGTCCATACCGTAAACG ATGAATGCTAAGTGTTGGAGGGTTTCCGCCCTTCAGTGCTGCAGCTAACGC ATTAAGCATTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGA ATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCT ACGCGAAGAACCTTACCAGGTCTTGACATACTATGCAAATCTAAGAGATTA GACGTTCCCTTCGGGGACATGGATACAGGTGGTGCATGGTTGTCGTCAGCT CGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATTAT CAGTTGCCAGCATTAAGTTGGGCACTCTGGTGAGACTGCCGGTGACAAACC GGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGC TACACACGTGCTACAATGGATGGTACAACGAGTTGCGAACTCGCGAGAGT AAGCTAATCTCTTAAAGCCATTCTCAGTTCGGATTGTAGGCTGCAACTCGC CTACATGAAGTCGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGA ATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGAGAGTTTGTA ACACCCAAAGTCGGTGGGGTAACCTTTTAGGAACCAGCCGCCTAAGGTGG GACAGATGATTAGGGTGAAGTCGTAACAAGGTAGCCGTAGGAGAACCTGC GGTTGGATCACC	97.78% *Lactobacillus plantarum* strain CIP 103151

4.3.5 Adhesion of the probiotics to the Caco-2 cells

For the determination of the adhesion capability to the human intestinal Caco-2 cell lines, all three isolates were selected to study their adhesion properties. The results showed that the isolates P10 exhibited the highest level of adherence (4.51%) followed by the isolate P11 (3.58%) as shown in Figure 4.8. Furthermore, both strains demonstrated the adhesion level superior to the reference probiotic strain, but this was not significant. Whereas, P09 showed a significantly lower level than the P10 isolate (1.78%). From this results, the P10 isolate showed the highest adhesion ability to the Caco-2 cells. Thus, the strains P10 was selected as candidate probiotic strains for development of synbiotic powder in the next section of the research.



* Represent a significantly different between group ($p<0.05$) by Tukey test.

Figure 4.8 The adhesion ability to Caco-2 cells of the isolates P09, P10, P11, and reference strains (*Lactobacillus casei* TISTR 1341 and *L. plantarum* TISTR 541)

4.3.6 Co-culture of the probiotic strains and bacterial pathogens

According to the previous experiment, *L. plantarum* P10 exhibited the highest adhesion *ability* to the Caco-2 cells that was one criterion of a good probiotic for health benefits. Therefore, the strain *L. plantarum* P10 was selected for the co-culture assay to investigate an antibacterial activity against 4 pathogenic bacteria: Gram negative (*E. coli* and *S. typhimurium*) and Gram positive (*B. cereus* and *S. aureus*). These four pathogens were food-borne bacteria that caused widespread food-borne illnesses and food poisoning.

L. plantarum P10 showed anti- *E. coli* activity by a significant decreasing of the *E. coli* population to 7.84 ± 0.16 log CFU/mL when compared with the *E. coli* in control group (8.91 ± 0.01 log CFU/mL). Moreover, there was a significant decrease ($P<0.05$) in the pH value in *L. plantarum* P10 co-culture with *E. coli* (4.82±0.01) when compared with the *E. coli* in the control group (4.98±0.01) at 24 hours of the incubation time (Figure 4.9).

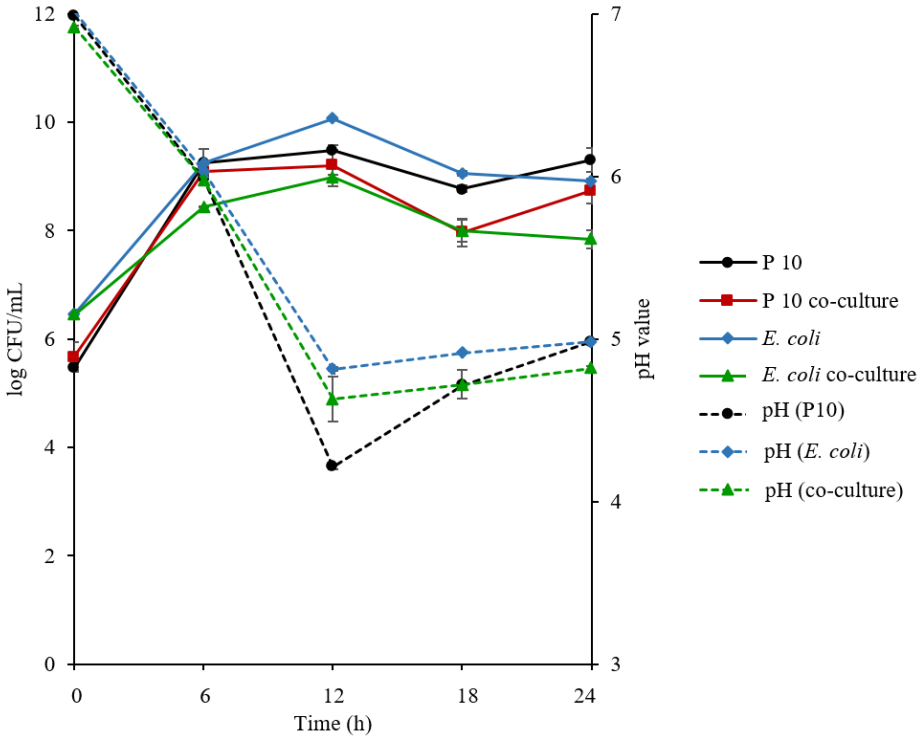


Figure 4.9 Results of co-culture assay of *L. plantarum* P10 and *E. coli*

Table 4.9 Inhibition effect of *E. coli* by *L. plantarum* P10 with co-culture assay

Incubation time (h)	*L. plantarum* P10 co-culture with *E. coli* (log CFU/mL)			
	L. plantarum P10	*L. plantarum* P10 co-culture	*E. coli*	*E. coli* co-culture
0	5.47±0.07	5.68±0.26	6.46±0.09	6.45±0.08
6	9.25±0.02	9.09±0.07	9.25±0.25*	8.44±0.01#
12	9.48±0.01	9.20±0.38	10.06±0.01*	8.98±0.04#
18	8.77±0.06[a]	7.97±0.26[b]	9.05±0.04*	8.00±0.21#
24	9.30±0.22	8.73±0.23	8.91±0.01*	7.84±0.16#

Note: Each value is cell count (log CFU/mL) represent the mean of determinations ± standard deviation. Statistical analysis was performed separately for activity of P10 against each pathogen. The different superscripts in a row at the same time period of P10/P10 co-culture and pathogen/pathogen co-culture indicates statistically significant differences ($p < 0.05$) as determined by Independent t-test.

Table 4.10 pH value during co-culture of *E. coli* with *L. plantarum* P10

Incubation time (h)	pH (mean ± SD)		
	***L. plantarum* P10**	***E. coli***	**Co-incubation**
0	6.99 ± 0.01^{a}	7.01 ± 0.01^{b}	6.92 ± 0.01^{c}
6	$5.98\pm0.01^{a,d,e}$	$6.03\pm0.01^{b,c,f}$	$5.98\pm0.03^{a,d,e}$
12	$4.21\pm0.02^{a,d,f}$	$4.81\pm0.01^{b,c,e}$	$4.63\pm0.14^{b,c,e}$
18	$4.71\pm0.01^{a,d,e}$	$4.91\pm0.01^{b,c,f}$	$4.72\pm0.09^{a,d,e}$
24	$4.98\pm0.01^{a,c,f}$	$4.98\pm0.01^{a,c,f}$	$4.82\pm0.01^{b,d,e}$

Note: The equal superscript lowercase letters in the same row indicate no significant differences ($p>0.05$) by Tukey test.

L. plantarum P10 co-culture with *S. typhimurium* displayed a significant decreased of *S. typhimurium* to 6.35±0.01 log CFU/mL when compared with the *S. typhimurium* in control group (8.41 ± 0.47 log CFU/mL). Moreover, there was a significant decrease ($p<0.05$) in the pH value when *L. plantarum* P10 co-culture with *S. typhimurium* (3.97±0.01) compared with the *S. typhimurium* culture in control group (4.92±0.00) at 24 hours of the incubation time (Figure 4.10).

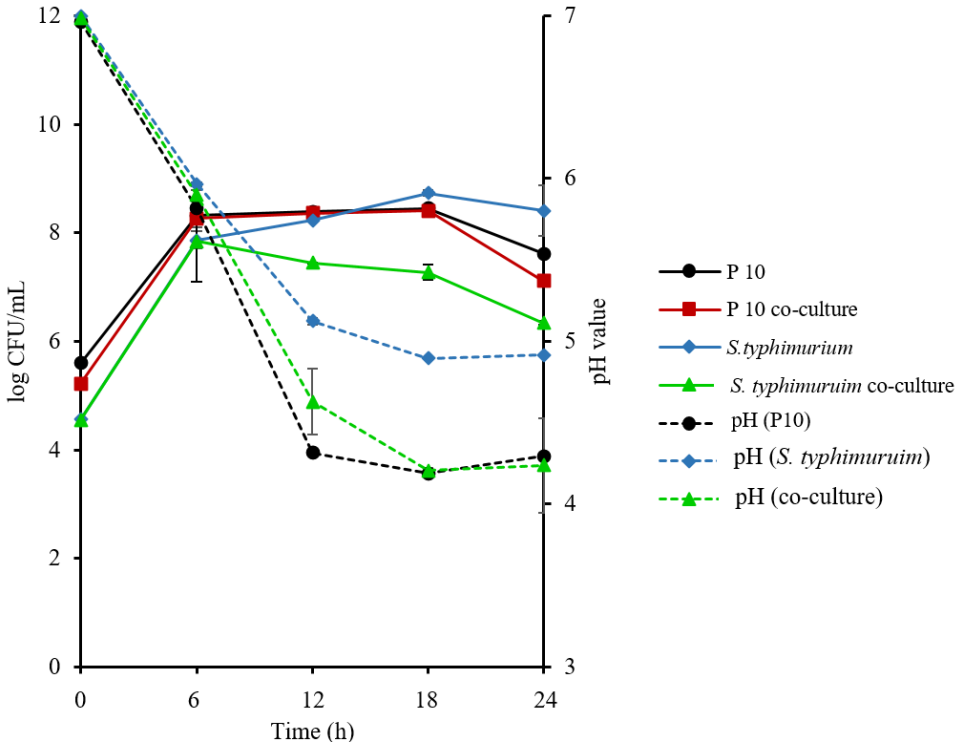


Figure 4.10 Results of co-culture assay of *L. plantarum* P10 and *S. typhimurium*

Table 4.11 Inhibition effect of *S. typhimurium* by *L. plantarum* P10 tested by co-culture assay

Incubation time (h)	*L. plantarum* P10 co-culture with *S. typhimurium* (log CFU/mL)			
	L. plantarum P10	*L. plantarum* P10 co-culture	*S. typhimurium*	*S. typhimurium* co-culture
0	5.59±0.04[a]	5.22±0.05[b]	4.56±0.02	4.55±0.02
6	8.33±0.11	8.27±0.16	7.87±0.16	7.85±0.75
12	8.39±0.05	8.37±0.03	8.23±0.02[*]	7.44±0.00[#]
18	8.45±0.01	8.42±0.01	8.73±0.05[*]	7.27±0.14[#]
24	7.62±0.00[a]	7.12±0.00[b]	8.41±0.47[*]	6.34 ±0.03[#]

Note: Each value is cell count (log CFU/mL) represent the mean of determinations ± standard deviation. Statistical analysis was performed separately for activity of P10 against each pathogen. The different superscripts in a row at the same time period of P10/P10 co-culture and pathogen/pathogen co-culture indicates statistically significant differences ($p < 0.05$) as determined by Independent t-test.

Table 4.12 pH value during co-culture of *S. typhimurium* with *L. plantarum* P10

Incubation time (h)	pH (mean ± SD)		
	***L. plantarum* P10**	***S. typhimurium***	**Co-incubation culture**
0	6.96±0.02[a]	7.00±0.03[a]	6.99±0.02[a]
6	5.82±0.02[a,d,f]	5.97±0.01[b,c,f]	5.90±0.03[b,d,e]
12	4.31±0.01 [a,d,f]	5.12±0.02 [a,d,f]	4.63±0.20 [a,d,f]
18	4.19±0.01[a,d,f]	4.90±0.01[b,c,f]	4.21±0.01[b,d,e]
24	4.29±0.01[a,d,e]	4.92±0.00[b,c,f]	4.24±0.29[a,d,e]

Note: The equal superscript lowercase letters in the same row indicate no significant differences (p>0.05) by Tukey test.

L. plantarum P10 co-culture with *B. cereus* showed a significant decreasing (p<0.05) of *B. cereus* to 7.25±0.04 log CFU/mL when compared with the *B. cereus* control group (log CFU/mL 7.54±0.02) Moreover, there was a significant decrease of the pH value in the P10 co-culture with *B. cereus* (4.08±0.05) when compared with the *B. cereus* control group (4.94±0.01) at 24 hours (Figure 4.11).

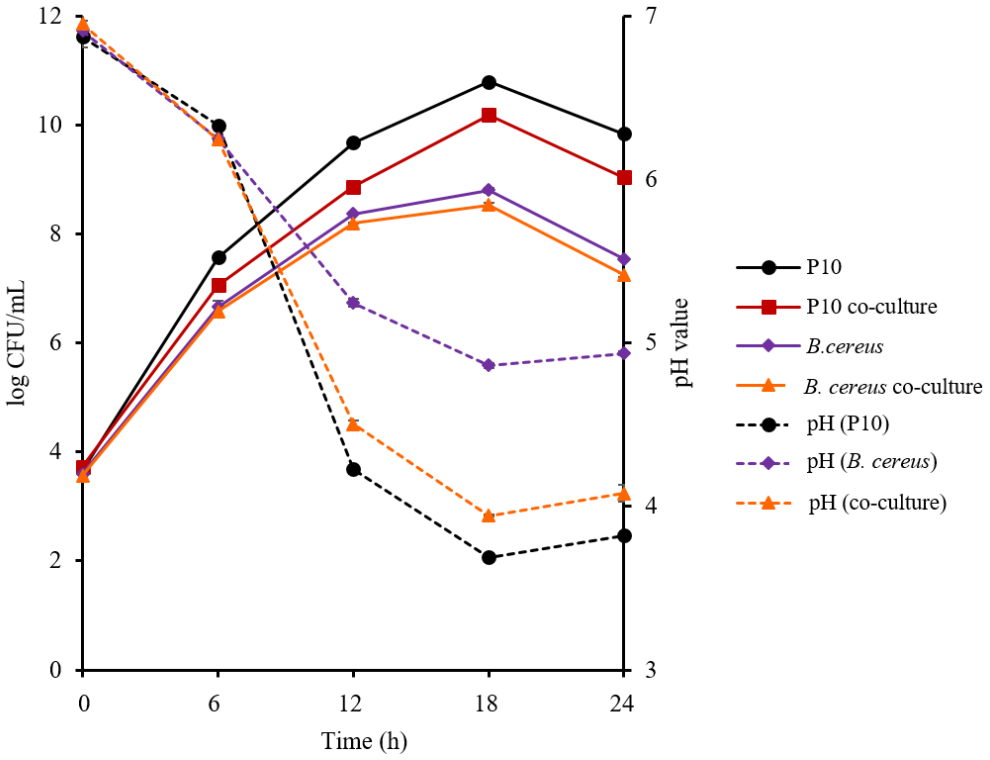


Figure 4.11 Results of co-culture assay of *L. plantarum* P10 and *B. cereus*

Table 4.13 Inhibition effect of *B. cereus* by *L. plantarum* P10 with co-culture assay

Incubation time (h)	*L. plantarum* P10 co-culture with *B. cereus* (log CFU/mL)			
	L. plantarum P10	*L. plantarum* P10 co-culture	*B. cereus*	*B. cereus* co-culture
0	3.66±0.05	3.73±0.04	3.60±0.09	3.56±0.04
6	7.57±0.05^{a}	7.06±0.09^{b}	6.66±0.12	6.58±0.08
12	9.68±0.03^{a}	8.87±0.06^{b}	8.38±0.01*	8.20±0.01$^{\#}$
18	10.80±0.05^{a}	10.19±0.05^{b}	8.80±0.05*	8.53±0.05$^{\#}$
24	9.84±0.02^{a}	9.04±0.06^{b}	7.54±0.02*	7.25±0.04$^{\#}$

Note: Each value is cell count (log CFU/mL) represent the mean of determinations ± standard deviation. Statistical analysis was performed separately for activity of P10 against each pathogen. The different superscripts in a row at the same time period of P10/P10 co-culture and pathogen/pathogen co-culture indicates statistically significant differences ($p< 0.05$) as determined by Independent t-test.

Table 4.14 pH value during co-culture of *B. cereus* with *L. plantarum* P10

Incubation time (h)	pH (mean ± SD)		
	***L. plantarum* P10**	***B. cereus***	**Co-incubation**
0	6.87±0.07[a]	6.91±0.00[a]	6.95±0.02[a]
6	6.33±0.01[a,d,f]	6.25±0.01[b,c,e]	6.24±0.01[b,c,e]
12	4.23±0.01[a,d,f]	5.25±0.02[b,c,f]	4.50±0.02[b,d,e]
18	3.69±0.00 [a,d,f]	4.86±0.02 [b,c,f]	3.94±0.01[b,d,e]
24	3.82±0.01 [a,d,f]	4.94±0.01 [b,c,f]	4.08±0.05 [b,d,e]

Note: The equal superscript lowercase letters in the same row indicate no significant differences ($p>0.05$) by Tukey test.

L. plantarum P10 co-culture with *S. aureus* showed a significant decreasing ($p<0.05$) of *S. aureus* to 2.47±0.07 log CFU/mL when compared with the *S. aureus* control group (8.97±0.02 log CFU/mL). Moreover, there was a significant decrease ($p<0.05$) of the pH value in the P10 co-culture with *S. aureus* (3.87±0.02) when compared with the *S. aureus* control group (4.86±0.01) at 24 hours (Figure 4.12).

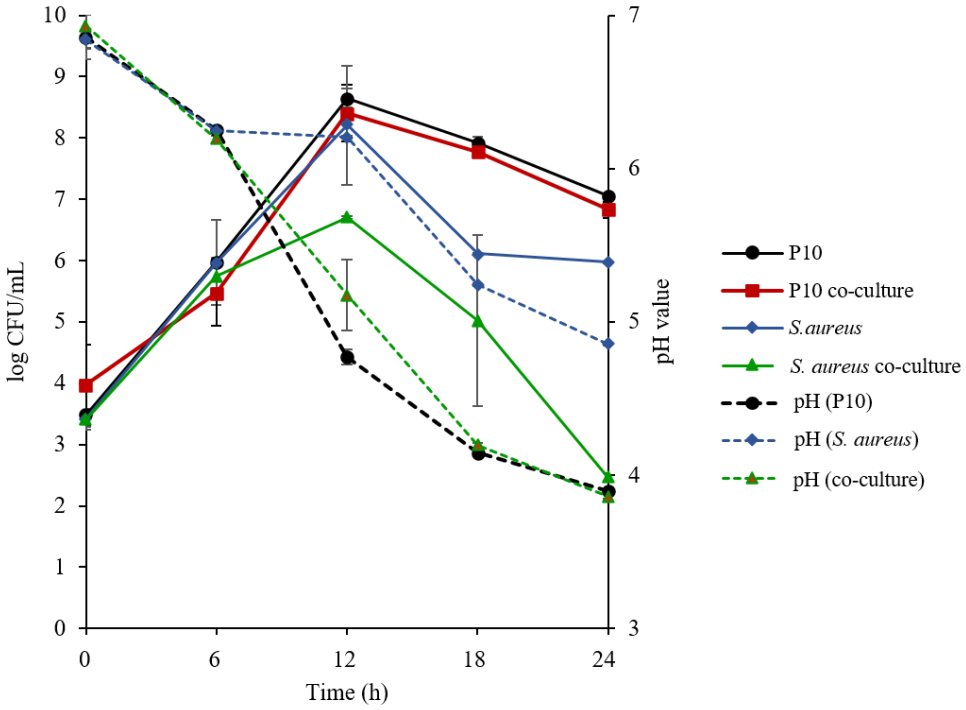


Figure 4.12 Results of co-culture assay of *L. plantarum* P10 and *S. aureus*

Table 4.15 Inhibition effect of *S. aureus* by *L. plantarum* P10 with co-culture assay

Incubation time (h)	*L. plantarum* P10 co-culture with *S. aureus* (log CFU/mL)			
	***L. plantarum* P10**	***L. plantarum* P10 co-culture**	***S. aureus***	***S. aureus* co-culture**
0	3.48±0.05	3.98±0.65	3.42±0.14	3.41±0.18
6	5.97±0.70	5.47±0.54	5.96±0.03	5.75±0.30
12	8.65±0.16	8.40±0.46	8.21±0.97	6.70±0.02
18	7.92±0.10	7.78±0.10	6.11±0.02	5.02±1.40
24	7.05±0.03	6.83±0.13	5.97±0.02*	2.47±0.07$^{\#}$

Note: Each value is cell count (log CFU/mL) represent the mean of determinations ± standard deviation. Statistical analysis was performed separately for activity of P10 against each pathogen. The different superscripts in a row at the same time period of P10/P10 co-culture and pathogen/pathogen co-culture indicates statistically significant differences ($p<0.05$) as determined by Independent t-test.

Table 4.16 pH value during co-culture of *S. aureus* with *L. plantarum* P10

Incubation time (h)	pH (mean ± SD)		
	***L. plantarum* P10**	***S. aureus***	**Co-incubation culture**
0	6.86 ± 0.15^{a}	6.84 ± 0.05^{a}	6.93 ± 0.15^{a}
6	$6.25\pm0.01^{a,c,f}$	$6.25\pm0.01^{a,c,f}$	$6.19\pm0.02^{b,d,e}$
12	$4.77\pm0.05^{a,d,e}$	$6.20\pm0.01^{b,c,f}$	$5.17\pm0.23^{a,d,e}$
18	$4.15\pm0.00^{a,d,f}$	$5.24\pm0.01^{b,c,f}$	$4.19\pm0.01^{b,d,e}$
24	$3.90\pm0.01^{a,d,e}$	$4.86\pm0.01^{b,c,f}$	$3.87\pm0.02^{a,d,e}$

Note: The equal superscript lowercase letters in the same row indicate no significant differences ($p>0.05$) by Tukey test.

4.4 Development of freeze dried synbiotic powder

According to the results of potential probiotic characterization, *L. plantarum* P10 exhibited appropriate properties to be the candidate probiotic strain for the development of synbiotic powder. This development was performed by a freeze-drying method with different cryoprotectants (skim milk and maltodextrin). The synbiotic powder was examined for the viability under simulation of GI tract conditions and the stability during storage at refrigerated and room temperature were also investigated by means of cell viability and antibacterial activity.

4.4.1 Preparation of freezed dried synbiotic powder

The probiotic candidate *L. plantarum* P10 was selected to use as probiotic starter for development of synbiotic powder. Freeze dried synbiotic powder was prepared by combining the *L. plantarum* P10 culture with inulin extracted from Jerusalem Artichoke together with skim milk or maltodextrin as cryoprotectant. The viability of *L. plantarum* P10 in the freeze dried synbiotic powder that using skim milk had a slightly decrease of viable cell count from 9.98± 0.26 to 9.94 ±0.41 Log CFU/mL, whereas in the synbiotic powder using maltodextrin, the viable cell count decreased from 9.87 ± 0.12 to 8.11 ±0.08 Log CFU/mL.

4.4.2 Survivability of synbiotic powder containing *L. plantarum* P10 under simulated gastrointestinal tract conditions

An important step toward the selection of potential probiotic candidates was to evaluate their resistance to the extreme conditions of the GI tract. The first barrier that had to be overcome was the oral cavity with a high concentration of enzymes in the human saliva then the stomach with low pH and digestive enzymes, and the upper intestine, which contained bile.

The results showed the viable cell counts after exposure to the amylase enzyme pH 6.75 for 10 minutes. The viable cell counts of synbiotic with skim milk were 9.86±0.05 Log CFU/mL and were 9.95±0.01 Log CFU/mL for the synbiotic sample formulated with maltodextrin. After incubated in the simulated gastric juice pH 2.5 for 60 minutes, the viable cell counts of synbiotic with skim milk were 8.01±0.01 Log CFU/mL and were 8.97±0.01 Log CFU/mL for the sample with maltodextrin. At the step of exposure to simulated intestinal juice pH 7.0 for four hours, the viable cell counts of synbiotic with skim milk were 7.82±0.05 Log CFU/mL and the lower cell viability was observed in the symbiotic powder prepared with maltodextrin at the viable cell count about 7.12±0.02 Log CFU/mL (Table 4.17). The results suggested that synbiotic with skim milk showed higher cell viability than the sample of maltodextrin. Therefore, synbiotic powder formulated with skim milk was selected for further stability test by evaluation of cell viability and its antibacterial activity during storage.

Table 4.17 Survivability of synbiotic powder containing *L. plantarum* P10 under simulated gastrointestinal tract conditions

Synbiotic powders	Bacterial counts (log CFU/mL) (% Survival)			
	Baseline	**Alpha-amylase enzyme**	**Simulated gastric juice**	**Simulated intestinal**
		10 min	**60 min**	**4 h**
Synbiotic with skim milk Exposed to the simulated gastrointestinal conditions	9.94 ±0.39^{a,c,f,h}	9.86±0.15(99.13%)^{a,c,f,h,A}	8.01±0.01(80.55%)^{b,d,e,g,A}	7.82±0.05(78.63%)^{b,d,e,g,A}
Control group (Synbiotic powder in NaCl)	9.94 ±0.39^{a,d,e,g}	11.12±0.05 (111.81%)^{b,c,e,h,B}	10.35±0.01(104.08%)^{a,c,e,h,B}	9.26±0.02(93.11%)^{a,d,f,g,B}
Synbiotic with maltodextrin				
Exposed to the simulated gastrointestinal conditions	8.11 ±0.13^{a}	9.95±0.02(100.04%)^{b,A}	8.97±0.01(90.19%)^{c,A}	7.12±0.04(71.57%)^{d,A}
Control group (Synbiotic powder in NaCl)	8.11 ±0.13^{a,d,f,h}	9.95±0.06(100.08%)^{b,c,f,h,A}	9.32±0.00(93.76%)^{b,d,e,g,B}	9.53±0.00(95.79%)^{b,d,e,g,B}

Note: The equal superscript lowercase letters in the same row indicate no significant differences (p>0.05) by Tukey test.

4.4.3 Stability test of synbiotic powder

4.4.3.1 Survivability of synbiotic during storage

From this data, the synbiotic powder was tested for the stability of the product. The results showed that during the storage of the synbiotic powder at refrigerated temperature, the viable cell count declined from 9.94±0.41 to 9.03±0.06 Log CFU/mL. However, after storage at room temperature, the viable cell count decreased from 9.92±0.03 to 8.68 ± 0.05 Log CFU/mL (Table 4.18).

The probiotic populations remained high (>7 Log CFU/mL) throughout the storage. Even though the cell counts of the probiotic strains decreased during the storage, the minimum effective dose for beneficial health effects (10^6–10^9 CFU/mL) in the product (Vasiljevic and Shah, 2008) was maintained throughout the 12 weeks of storage.

Table 4.18 Stability test of the synbiotic powder during storage at refrigerated and room temperature for 12 weeks.

Storage time (Weeks)	Refrigerated temperature		Room temperature	
	Log CFU/mL (mean ± SD)	% Survival	Log CFU/mL (mean ± SD)	% Survival
0	9.94±0.41	-	9.92±0.03	-
1	9.91±0.07	99.71	9.87±0.10	99.50
2	9.61±0.35	96.68	9.03±0.04	91.01
3	9.89±0.01	99.49*	8.71±0.09	87.77*
4	9.75±0.18	98.05	8.61±0.03	86.79
8	9.14±0.28	91.98*	8.71±0.27	87.80*
12	9.03±0.06	90.82	8.68±0.05	87.54

Note: * Represent a significantly different between group ($p<0.05$) by Tukey test.

4.4.3.2 Anti-food-borne bacteria of the synbiotic during storage

The synbiotic powder was tested for the antimicrobial activity during storage at refrigerated and room temperature. Four pathogenic bacteria including Gram negative (*E. coli* and *S. typhimurium*) and Gram positive bacteria (*B. cereus* and *S. aureus*), which were food-borne bacteria that caused widespread food-borne illnesses and food poisoning were used as indicator strains.

As can be seen in Table 4.19, the synbiotic powder stored at refrigerated temperature showed antimicrobial activity against all the pathogenic bacteria after being stored for a period of 12 weeks. The synbiotic powder showed percentage inhibition of *E. coli, S. typhimurium, B. cereus and S. aureus* at 71.63%, 66.29%, 60.23% and 64.08%, respectively. However, the synbiotic powder stored at room temperature showed antimicrobial activity against all the pathogenic bacteria after being stored for a period of 8 weeks. The synbiotic powder showed percentage inhibition of *E. coli, S. typhimurium, B. cereus and S. aureus* at 103.15%, 77.78%, 82.42% and 71.03%, respectively.

Table 4.19 Antimicrobial activity of synbiotic powder during storage at refrigerated and room temperature for 12 weeks

Weeks	Storage	Inhibition zone (mm; mean± SD)			
		Gram negative bacteria		Gram positive bacteria	
		E. coli	*S. typhimurium*	*B. cereus*	*S. aureus*
0	Refrigerated temp.	13.50±1.05[a]	14.33±1.51[a]	13.83±0.75[a]	13.67±1.37[a]
	Room temp.	13.33±1.03[a]	13.50±2.17[a]	11.83±0.75[a]	12.67±1.75[a]
	Tetracycline (50 µg/mL)	20.17±1.60	21.00±0.63	22.00±1.55	25.33±0.82
1	Refrigerated temp.	13.17±0.98[a,c,f,h]	13.33±1.37[a,c,f,h]	10.67±1.97[b,d,e,h]	12.83±0.75[a,c,e,g]
	Room temp.	12.83±0.75[a]	12.67±1.97[a]	10.33±1.63[a]	11.67±1.86[a]
	Tetracycline (50 µg/mL)	21.17±0.75	20.67±0.82	21.67±1.51	25.17±0.98
2	Refrigerated temp.	10.67±0.52[a]	11.83±1.72[a]	10.67±1.37[a]	12.00±1.79[a]
	Room temp.	11.33±0.82[a]	12.67±1.21[a]	11.67±1.37[a]	11.67±1.37[a]
	Tetracycline (50 µg/mL)	19.50±1.05	21.50±0.84	27.50±1.64	20.83±1.60

Note: The equal superscript lowercase letters in the same row indicate no significant differences ($p>0.05$) by Tukey test.

Table 4.19 Antimicrobial activity of synbiotic powder during storage at refrigerated and room temperature for 12 weeks. (Continued)

Weeks	Storage	Inhibition zone (mm; mean± SD)			
		Gram negative bacteria		Gram positive bacteria	
		E. coli	*S. typhimurium*	*B. cereus*	*S. aureus*
3	Refrigerated temp.	$12.17\pm1.33^{a,d,e,f}$	$9.67\pm0.52^{b,c,e,f}$	$11.17\pm1.83^{a,c,e,f}$	$11.00\pm1.10^{a,c,e,f}$
	Room temp.	10.83 ± 1.17^{a}	11.17 ± 0.75^{a}	10.83 ± 1.17^{a}	10.50 ± 1.64^{a}
	Tetracycline (50 µg/mL)	19.67±1.37	20.17±1.17	27.17±1.17	26.00±1.10
4	Refrigerated temp.	$14.00\pm1.41^{a,d,f,g}$	$10.50\pm0.84^{b,c,e,h}$	$11.00\pm0.89^{b,c,e,h}$	$14.83\pm1.33^{a,d,f,g}$
	Room temp.	$13.50\pm1.29^{a,d,f,h}$	$10.50\pm0.55^{b,c,f,g}$	$12.17\pm1.33^{a,c,e,g}$	$11.00\pm1.67^{b,c,f,g}$
	Tetracycline (50 µg/mL)	19.75±1.26	21.67±0.82	28.33±1.03	22.67±0.82
8	Refrigerated temp.	$14.50\pm1.29^{a,c,f,h}$	$12.25\pm0.96^{a,c,e,h}$	$9.75\pm1.50^{b,c,e,g}$	$9.50\pm1.00^{b,d,e,g}$
	Room temp.	$13.75\pm0.96^{a,d,f,h}$	$10.50\pm1.00^{b,c,e,g}$	$9.75\pm0.50^{b,c,e,g}$	$9.00\pm0.00^{b,c,e,g}$
	Tetracycline (50 µg/mL)	19.75±1.26	21.75±1.26	27.25±0.96	24.50±2.65

Note: The equal superscript lowercase letters in the same row indicate no significant differences ($p>0.05$) by Tukey test.

Table 4.19 Antimicrobial activity of synbiotic powder during storage at refrigerated and room temperature for 12 weeks. (Continued)

Weeks	Storage	Inhibition zone (mm; mean± SD)			
		Gram negative bacteria		Gram positive bacteria	
		E. coli	***S. typhimurium***	***B. cereus***	***S. aureus***
12	Refrigerated temp.	9.67±0.82[a]	9.50±1.52[a]	8.33±0.52[a]	8.33±0.52[a]
	Room temp.	0±0	0±0	0±0	0±0
	Tetracycline (50 µg/mL)	21.00±0.89	21.67±1.03	28.17±0.98	26.00±1.10

Note: The equal superscript lowercase letters in the same row indicate no significant differences ($p>0.05$) by Tukey test.

CHAPTER 5
DISCUSSION AND CONCLUSION

The objectives of this study aimed to isolate and characterize lactic acid bacteria (LAB) obtained from fermented foods for investigating the probiotic properties and development of the synbiotic powder. Eighty-three LAB isolates were screened for antibacterial activities against food–borne pathogens. Ten isolates of LAB that showed the highest antibacterial activity against each indicator pathogen were chosen to determine the acid and bile salt tolerances. Three LAB isolates (P09, P10 and P11) were selected for further characterization according to the demonstration of high level of acid and bile salt tolerance. The antibacterial activity of neutralized cell-free supernatant was performed to verify the antibacterial substance produced by LAB. According to the Generally Regarded As Safe (GRAS) criteria for using probiotics, probiotic strain must be considered for safety to human host. Thus the potential LAB isolates were identified by 16S rDNA gene analysis and tested for antibiotic susceptibility, hemolytic and DNase activity. For selection of good probiotic, the strain must be capable of exerting a beneficial effect on the hosts and adhere to intestinal epithelial tissue. The *in vitro* adhesion assay of probiotic strain demonstrated that the strain *L. plantarum* P10 showed the highest adhesion ability to Caco-2 cell line and also exhibited significant inhibitory effects against four foodborne bacteria by co-culture assay. The strain *L. plantarum* P10 was then selected to develop the synbiotic powder combining with inulin extracted from JA by freeze-drying method. The freeze dried synbiotic powder showed the high level of survivability under simulated gastrointestinal conditions. Additionally, this synbiotic powder could achieve the desirable cell viability and anti-foodborne pathogens activity during storage at refrigerated and room temperatures for 12 weeks.

5.1 Isolation and characterization of LAB from fermented foods

The total of 83 isolates of lactic acid bacteria (LAB) isolated from several fermented foods were tested for antibacterial activity for primary screening of the potential probiotic strains. Many studies also reported the LAB presenting in various traditional foods such as Thai fermented fish (Paludan-Müller, Henrik Huss and Gram, 1999) Pla-Som-

Fug (Deatraksa et al., 2018) Pla-som (Sanpa, Sanpa and Suttajit, 2019) and Thai fermented meat and fish products (Pringsulaka et al., 2012). Morphological characteristics of the LAB colonies were circular, creamy, and smooth with an entire margin. LAB represented cocci and rod-shaped cells and all 83 LAB isolates were Gram-positive and catalase-negative bacteria. LAB probiotics are mainly gram-positive, anaerobic, non-sporulating, and acid tolerant bacteria that can ferment a variety of nutrients primarily into lactic acid. LAB are microflora present in most of the traditional fermented foods and starter cultures of LAB that are industrially important in food safety (Sathe and Mandal, 2016). These are desirable microflora of the gastrointestinal tract (GIT) and are thus generally regarded as safe (GRAS) (Tannock, 1997). LAB are involved in the fermentation and are the dominant microflora of fermented products. They are known to play an essential role in food preservation and inhibit spoilage microorganisms or food-borne pathogens by the production of lactic acid, acetic acid, H_2O_2, bacteriocin, diacetyl, and CO_2 (Nur and Aslim, 2010). The antimicrobial effect of LAB is mainly due to their lactic and organic acid production, causing the pH of the growth environment to decrease. Low pH induces organic acids to become lipid soluble and to diffuse through the cell membrane into the cytoplasm. LAB also produces acetaldehyde, hydrogen peroxide, diacetyl, carbon dioxide, polysaccharides and bacteriocins, that have an important role in antimicrobial activity (Kuipers, Buist and Kok, 2001).

5.2 Screening of LAB for probiotic properties

The total of 83 LAB isolates were primarily screened for probiotic properties. Antibacterial activities against six foodborne pathogens were examined and 10 LAB isolates were chosen to determine for the acid and bile salt tolerance.

5.2.1 Screening for antibacterial activity of LAB

All 83 LAB isolates obtained in this study demonstrated a strong inhibitory effect against *S. aureus, P. aeruginosa*, *S. enteritidis*, *E. coli*, *S. typhimurium,* and *B. cereus.* There are 51 LAB isolates showed the inhibitory activity against all six bacterial pathogens. Moreover, the isolates FFPR2, FFPS1, P01, P02, P03, P05, P06, P09, P10, and P11 showed the highest inhibition zone against each pathogenic bacteria.

LAB has produced many organic acid compounds resulting in the decreasing of pH levels that could inhibit unfavorable bacteria, including pathogenic and spoilage bacteria. Inhibition of pathogens is a major probiotic selection criterion and this mechanism is involved in the restoration of gut microbiota balance. The obtained results are in agreement with the previous studies showing probiotic antimicrobial activity against target pathogenic bacteria by *L. fermentum* strains which can inhibit *E. coli* ATCC25922, *P. aeruginosa* ATCC27853, *S. aureus* ATCC29213 and *K. pneumonia* ATCC25656 by agar well diffusion assay (Tulumoğlu, Kaya and Şimşek, 2014).

5.2.2 Acid and bile salt tolerance

Ten selected LAB isolates were tested for the low pH resistance and examined the effect of bile salts tolerance. Three LAB isolates; P09, P10 and P11 were selected with consideration to their tolerance to high acid at pH 2 and 3 and high bile salt resistance at 0.15% and 0.30% w/v. The results demonstrated that LAB isolates can survive through the digestive systems. The ability of an organism to survive under acidic conditions (low pH) and bile salts environments is essential and required for probiotics. Lee et al. (2016) reported that *Lactobacillus plantarum* C182 survived well after 3 h exposure to 0.30% bile salts with the survival ratio of 58.53 %.

In this study, the survival rates of all 10 isolates decreased when exposed to 0.3% bile salts except the isolates P06, P09, P10, and P11. The resistance to low pH conditions of LAB isolates is crucial to survive under human gastrointestinal (GI) conditions. The strains that considered to use as probiotics must exhibit a high GI tolerance. The strains used in this study showed great resistance to acid condition, thus this may be due to the fact of their origins in which they were isolated from high acid foods (fermented foods). According to their strong acid and bile salt tolerances (viability at pH 2.0 more than 100% and survivability at 0.15% bile salt higher than 120%), the 3 LAB isolates P09, P10 and P11 were then selected for further investigation of Caco-2 cells adhesion.

In the human GI tract condition, the mean bile concentration is believed to be 0.3% w/v, a concentration considered as critical and sufficient to screen for bile tolerance and resistance. Some studies reported that *Lb. plantarum* strains remained viable in the presence of 1.8 % bile. This is particularly evident after a meal, when bile salt concentrations can increase sharply in the duodenum, and in the ileum where the

concentration falls below 4 mmol/L due to active ileal. *Lb. plantarum* can survive one of the main gastrointestinal stresses. Thus, it suggested that all the strains should be viable and reaching the duodenum and in GI transit. Microbial bile tolerance is a recognized criterion for probiotic strain selection (Prete et al., 2020).

Todorov et al. reported that the maximum growth of *Lactobacillus plantarum* ST16P isolated from papaya was observed at pH 4.0 and pH 5.0 *Lb. plantarum*, *Lb. rhamnosus*, *Lb. pentosus*, *Lb. paracasei* exhibited growth suppression at pH 3.0 and pH 4.0. Previous researchers reported that various hydrogen ion concentrations affect the growth of bacteria and suppressed bacterial growth (Todorov et al., 2011). LAB strains have been reported for the survivability in the plates containing 0.3 % bile salts (Han et al., 2017).

Guo et al. demonstrated that the viable counts of most LAB from swine feces and intestines were not reduced after 3 h exposure at pH 3.0 but significantly reduced after 3 h exposure at pH 2.0. This result suggested that the tolerance of LAB to acid was strain-specific. Moreover, 0.3% bile salts did not show any killing effect on all isolates and most of them even grew in the presence of 1.0% bile salts. The probable reason of isolates showing high tolerance to bile was that the isolates were originated from animal feces and intestines and had more chances to be exposed to bile salts (Guo et al., 2010). However, in this study, the survival rates of all LAB isolates decreased when exposed to 0.3% bile salts except the isolates P09, P10, and P11. Thus, these 3 isolates exhibited the basic requirements of the potential probiotics and were then selected for the next experiments.

5.3 Characterization of selected potential probiotic strains

5.3.1 Antimicrobial activity of neutralized cell-free culture supernatant

No inhibition was observed when neutralizing supernatants to the pH 6.5 was tested for antibacterial activity. Therefore, inhibitory effects cannot be explained by bacteriocin action and were most probably due to the production of organic acids along with the low pH. Similarly, a previous study also found that antimicrobial activity of lactic acid bacteria from swine origins was significantly decreased after neutralization, and no inhibition against the pathogens was observed when the pH was adjusted to 6.0. The difference of inhibitory activity under the same pH condition might be caused by

some undissociated acids or some unknown antimicrobial substances (such as bacteriocins) (Guo et al., 2010).

5.3.2 Safety test

The antibiotic susceptibility test, hemolytic test and DNase test of the selected LAB isolates were performed according to safety criteria on human host.

Antibiotics are a major tool utilized by the medical industries to fight pathogens. However, antibiotic resistance can cause significant danger and suffering for many people with pathogen infections. Thus, probiotics should be sensitive to commonly prescribed antibiotics at low concentrations. The previous study (Wook et al., 2016) showed that four LAB strains were tested for antibiotic susceptibilities and they were sensitive to ampicillin, chloramphenicol, cyclohexamide, erythromycin, neomycin, streptomycin, tetracycline, and rifampicin. Nevertheless, all strains were resistant to vancomycin at the highest amount (2048 µg.) *Leu. mesenteroides* C4 and *Leu. mesenteroides* C10 were resistant to spectinomycin. *L. plantarum* strains were found to be susceptible to ampicillin, and it showed resistance to vancomycin. The vancomycin resistance observed among *Lb. plantarum* strains were interpreted to represent the fact that the majority of the lactobacilli were intrinsically resistant to glycopeptide (Wook et al., 2016).

Besides, *Lactobacillus* strains isolated from infant feces were resistant to kanamycin and streptomycin. All 21 *Oenococcus onei* strains isolated from wine showed resistance to vancomycin, indicating the intrinsic resistance. The susceptibility and resistance of LAB to various antibiotics are variable, depending on the species. One thing to be cautious about the selection and characterization of a probiotic is antimicrobial resistance. Previous studies also confirm the generally lower resistance of the *lactobacilli* species studied here towards tetracycline and chloramphenicol. It is generally known that fermented foods containing pathogenic microbes transfer their resistant genes to other microbes existing in the gut of human microflora. Zhou et al. reported that *Pediococcus, Lueconostoc,* and *Lb. rhamnosus* showed resistance to vancomycin, kanamycin, and tetracycline. On the other hand, reported that antibiotic-resistant strains involved in antibiotic induced diarrhea (Zhou et al., 2005). Therefore, the test of antibiotic susceptibility is necessary to examine before using probiotics for health. There is possibility of transferring of antibiotic resistant genes to other

pathogenic bacteria, but the norfloxacin resistance in lactobacilli have been reported to be intrinsic, chromosomally encoded and not transferable (Bernardeau et al., 2008; Varankovich et al., 2015). Therefore, those probiotic strains with intrinsic antibiotic resistance could be restoring the intestinal microbiota after antibiotic treatment. The antibiotic resistance of the strain P09 to gentamicin and sulphamethoxazole may require the further investigation to investigate the possibility of resistant gene transferring.

According to FAO guidelines, microbial strains to be used as probiotics are recommended to be safe in the host. The selection and application of strains devoid of haemolytic activity can be used as probiotics. Results showed that all three isolates did not show any red blood cell digestion and DNase activity. Therefore, all isolates may be considered as a safe prerequisite for the selection of the probiotic strain used in food matrices. Similar results indicating that the majority of LAB strains are non-haemolytic (Tejero-Sariñena et al., 2012) (Bautista-Gallego et al., 2013) (Boricha et al., 2019). LAB was also reported to be lack of deoxyribonuclease (Gupta and Malik, 2007).

5.3.3 LAB Identification

The isolates P09, P10 and P11 were identified as *Lactobacillus plantarum* at the similarity at (94.79%, 95.52%, and 97.78%, respectively. Paludan-Müller and colleagues in 2002 had also reported that *Lactobacillus plantarum* had been isolated from Thai fermented fish (Paludan-Müller et al., 2002). *L. platarum* strains as members of probiotic strains are generally regarded as safe and they have a long history of use.

5.3.4 Adhesion of probiotic to Caco - 2 cells

The adhesion capacity of LAB has been considered as an important property of probiotics. The selection criterion for a potential probiotic is their ability to adhere to the intestinal mucosa for colonization of the gut for optimal functionality. This ability may provide beneficial effects, such as the exclusion of pathogens. Specifically, the human intestinal Caco - 2 cell line is widely used in assays to evaluate the adhesion properties of potential probiotic strains with *in vitro* models because features of Caco-2 cells are similar to those of mature enterocytes. (Sambuy et al., 2005). In these results, the isolate P10 presented the highest adhesion ability (4.52%). Adhesion levels of enological LAB to Caco-2 cells varied from 0.37% to 12.2%, depending on the strain, species and genera. This dependence is in line with a previous study (Collado et al., 2006), whose adhesion values ranged from 0.9% (*Pediococcus freudenreichii* JS) to

20% (*Lactobacillus rhamnosus* GG). *P. pentosaceus* CIAL-86 presented the highest adhesion percentage (12.2%), followed by *L. plantarum* CIAL-121 (7.10%), both with adhesion values superior to the reference probiotic strain.

The adhesion ability is affected by many factors, including the production of exopolysaccharide (EPS). The structure of EPS may promote strain-specific interactions of bacteria with specific receptors and effectors of Caco-2 cells (Ruiz et al., 2014). The study about *L. brevis* R4 showed the highest adhesion rate (23.0%) among the 10 LAB. The adhesion rates of the different LAB ranged from 0.8% to 23.0%, whereas *L. fermentum* showed the lowest adhesion rate of 0.8%. Adhesion of LAB to Caco-2 cells is strain-specific, varying within the same species. Adhesion of LAB is a complex process that involves contact between the bacterial cell membrane and interacting surfaces. These results show adhesion levels of LAB to Caco-2 cells varied from 1.78% to 4.52%.

Similarly, adhesion of lactobacilli has been claimed to be essential for the exertion of a beneficial (probiotic) effect in the large intestine. Previous studies have reported the study of probiotic potential of *Lactobacillus* strains isolated from dairy products which demonstrated that most of the strains tested had low adhesion to Caco-2 cells (<4%). Nine strains were found to adhere to Caco-2 cells with percentages ranging from 4.4% to 25.5%, with the highest values obtained for *L. plantarum* ACA-DC 146 (25.5%), and *L. paracasei* subsp. *paracasei* strains ACA-DC 221, 3334 and 3335 (13.1, 13.8 and 11.8%, respectively) (Maragkoudakis et al., 2006).

Additionally, the study on probiotic properties of *Lactobacillus* strain from fermented sausages showed that the *Lact. plantarum* strains AL2, AL5, CL5, CL9, DL6 and GL2, *Lact. paraplantarum* strain CL6 and *Lact. pentosus* strain FL2 adhered at the highest levels ($\geq 6.5x10^7$ CFU/well) (Pennacchia, Vaughan and Villani, 2006).

The adhesion level of *Lactobacillus plantarum* strain L15 to Caco-2 cells was 12%. Furthermore, this strain showed appropriate anti-adherence effects, including competition, inhibition, and replacement properties against *Escherichia coli*. The results indicated that *Lactobacillus plantarum* strain L15 had good potential for exerting antagonistic effects against *E. coli* (Alizadeh Behbahani, Noshad and Falah, 2019). Tallon et al. also showed the adhesion capacity of *L. plantarum* to Caco-2 cell (Tallon et al., 2007).

Mechanism adhesion of LAB is by receptor-specific binding with Val-der-Waals force (charge) and the binding of cells and receptors by hydrophobic interaction, which in general the bacterial cell walls have cell surface hydrophobicity causing the outer membrane hydrophobicity (van Loosdrecht et al., 1987). In addition, bacteria can generate protein on the cell wall (extracellular matrix molecules) such as collagen, fibronectin and vitronectin and by these proteins can adhere to the mucous membrane on the intestinal wall (Lorca et al., 2002).

The ability to adhere to the epithelial cells is one of the main criteria for selecting probiotic strains. This ability is regarded as a prerequisite to exert beneficial effects. Caco-2 cells are derived from colon carcinomas and represent the major cell phenotypes found in the human intestinal mucosa. *L. plantarum* P10 was able to adhere to Caco-2 cell. The results indicated that *L. plantarum* P10 adhered and colonized intestinal epithelium cells that can provide an inhibiting of pathogenic bacteria. *L. plantarum* P10 had significantly higher adhesive properties to Caco-2 cells than *L. plantarum* P09 but not significantly difference comparing to other strains. This may be due to the specificity of cell adherence. The mechanisms involved in protection against pathogen adhesion have been proposed to be by either non-specific hindrance of receptors for pathogens or competition with pathogens for the binding sites. This result indicates that the balance of normal probiotic *Lactobacillus* may provide a good protection against the adhesion of pathogens to epithelium cells (Kaewnopparat et al., 2013).

5.3.5 Co-culture of probiotic strains and bacterial pathogens

LAB has been studied and confer the inhibitory activity against a group of foodborne pathogens and reduces the growth of pathogenic microbes. The lowering in pH level was due to lactic acid production by LAB. The co-culture assay showed the suppression of both grams negative (*E. coli* and *S. typhimurium*) and gram positive (*B. cereus* and *S. aureus*) pathogenic bacteria after 24 h of co-incubation. This indicates that *L. plantarum* P10 may be useful in preventing and inhibiting the growth of pathogenic bacteria. Each pathogenic strain was co-cultured with a probiotic strain to study the antagonism. In our study, *L. plantarum* P10 had strong antimicrobial activity against 4 pathogenic bacteria agreeing with the previous work from the co-culture assay reported by Cheng-Chih et al., which reported the inhibition of *E. coli* growth by *Lactobacillus*

strains (Tsai, Lin and Hsieh, 2008) and a co-culture study in which *Lactobacillus* strains inhibited the *in vitro* growth of *E. coli, Salmonella enteriditis* was also reported by Drago et al., (1997). Szala et al. co-cultured of six *Lactobacillus* strains with *Salmonella senftenberg* and presented a report that all the tested strains inactivating the growth of pathogen during a 48 hours of co-culture (Szala, Paluszak and Motyl, 2012). According to the study by Tulumoglu et al. (2013) and also the results of the present study confirmed the strain specific nature of LAB isolates (Tulumoglu et al., 2013).

The use of probiotics has been recently proposed as a viable option for the prevention or treatment of *S. aureus* infectious diseases. *Lactobacillus acidophilus* and *L. casei* have an inhibitory effect on *S. aureus*, possibly by the combination of nutritional competition, secretion of antibacterial peptides, proteins or immunomodulation effect (Kang et al., 2017).

Additionally, the study of antagonistic effect of probiotic strains against pathogens showed that *Lactobacillus* strains reduced *Salmonella Typhimurium* greater inhibition than *E. coli* O157: H7 in the co-culture assay, ($p < 0.05$) at 16 h of incubation (Sanchez et al., 2013). Antimicrobial activity of *Lactobacillus* and *Bifidobacterium* strains against intestinal pathogens was due to the organic acid production, particularly lactic and acetic acids (Fooks and Gibson, 2002).

Another study demonstrated the antagonism of 15 *Bifidobacterium* strains (*B. animalis*, *B. globosum* and *B. breve*) against six *Salmonella* strains (*Salmonella enteritidis* and *Salmonella Typhimurium*). They found that all strains of *Bifidobacterium* were effectively antagonistic against *Salmonella* strains, which were fully inhibited before the end of exponential phase, then their viability was severely affected at the beginning of the stationary phase. The *Salmonella* strains CFU ranged from zero to 5.13 logCFU/ml. Growth of *Bifidobacterium* strains in co-cultures was similar to that observed in mono-cultures (Kailasapathy and Chin, 2000). Cheikhyoussef et al. (Cheikhyoussef, Pogori and Zhang, 2007), found that supernatants obtained from four bifidobacterial strains contained a proteinaceous antimicrobial compound which inhibited the growth of *Bacillus cereus* and *E. coli* AS 1.543.

The antimicrobial assay showed an enhanced inhibition at the lower pH may be due to an increasing efficacy of the inhibitory agent in the supernatant, or an additive effect of the inhibitory agent plus suboptimal pH. Lactic acid bacteria produce many

organic acids such as lactic, acetic and propionic. Gram negative pathogens, such as *E. coli* O157 and *E. coli* O86 tend to be more sensitive to organic acids than bacteriocins (Alakomi et al., 2000).

After 24 h incubation the pH value decreased from the beginning (0 h.) resulting from the acid producing by LAB. Some studies reported that the pH values decreased rapidly in the first 9 h and then decreased slowly, when the strain reaching the stationary phase. Usually, the growth of LAB reaches the stationary phase when the pH is less than 4.5 (Xu et al., 2018). The generation of massive amounts of organic acids (mainly lactic acid) and other metabolites by LAB might be responsible for the reductions in pH. Lactic acid and pH are known to play important roles in the antibacterial activity of LAB. Gao, Li, and Liu (2015) reported that bacteriostasis by metabolites generated by LAB is more efficient at a pH of less than 5.5. The acid-producing ability of LAB is also related to their heat stress response (Han et al., 2017).

A previous study reported that probiotic bacteria showed anti-foodborne Gram negative and gram positive bacteria, this is related to this study that *L. plantarum* P10 showed anti-foodborne pathogens. Therefore, the important features for probiotics screening are the production of antimicrobial compounds to compete for the pathogen survival in the intestinal tract and express the probiotic effect in their hosts. This finding confirms the anti-foodborne pathogens of LAB, which is an option to alternative treatment in prolong antibiotic use and resistance of pathogenic bacteria to antibiotics.

5.4 Development of synbiotic powder by freeze-drying method

5.4.1 Preparation of synbiotic powder

This study uses the probiotic strain *L. plantarum* P10 combined with inulin powder together with skim milk or maltodextrin as cryoprotective agents for synbiotic powder preparation by freeze-drying method.

5.4.2 Survival rates after freeze-drying and stability tests

The loss of viability at different storage periods was observed. The lower survival rates of *L. plantarum* P10 during storage may be related to the extensive damage that might have occurred as a result of the freeze-drying process. The survival rate of the culture during drying and following storage depends upon many factors, including the species and strain of the culture, the drying conditions, the inoculums and

supporting medium or carrier used, pre-adaptation of the culture to acquire resistance to processing conditions and the use of thermoprotective agents. The use of thermoprotective agents can help to improve the survivability of probiotic culture (Corcoran et al., 2004).

The study effect of buckwheat flour and oat bran on growth and cell viability of the probiotic strains *Lactobacillus rhamnosus*, *Lactobacillus paracasei* and their combination, in synbiotic fermented milk show the stability during storage at 4 °C for 28 days. This synbiotic fermented milk carries about 10^9 viable cells (Coman et al., 2013).

The results by Oliveira (Oliveira et al., 2011) for inulin in which an improvement of bacterial count of binary co-cultures and prebiotic cocktails in fermented skim milk was highlighted. These studies confirmed the already well known prebiotic effect of inulin for both *bifidobacteria* and *lactobacilli*. Viability of *lactobacilli* is affected because of several factors including acid produced during fermentation, oxygen content in the product and oxygen permeation through the packaging material. However, additional advantages can be obtained by protecting cells from injury during storage. For all these reasons it is important to follow the probiotics viability during manufacture and storage. The viable counts at the end of fermentation varied from 2.05×10^8 CFU/ml to 5.93×10^9 CFU/ml. Although slight CFU reductions occurred in some samples after 28 days of storage, all samples supplied the minimum 10 billion per portion amount of probiotics required and many samples were well above 10^9 CFU/ml (Coman et al., 2013).

The study by Angiolillo (Angiolillo et al., 2014) that formulated a synbiotic Fiordilatte cheese with an edible sodium alginate coating as a carrier of probiotic (*Lactobacillus rhamnosus*) and prebiotic fructo-oligosaccharide substances (FOS). Results proved that viability of lactic acid bacteria in the functional product remained over the imposed limit (10^7 CFU/g) for the entire storage period in all the experimental trials with bacterial counts about 4.52×10^7 CFU/g at 4 °C, 3.42×10^7 at 9 °C and 4.62×10^7 at 14°C. Furthermore, the addition of probiotic and prebiotic substances in the coating provoked a slight antimicrobial effect against *Pseudomonas* spp. and *Enterobacteriaceae* (Angiolillo et al., 2014).

A freeze-dried synbiotic formulation was prepared by incorporating *L. plantarum* and prebiotics using non fat dry milk as base material. The product contained high numbers of viable probiotics even after storage for 90 days at 4±1°C. Viable counts (8 to 9 log cfu/g) of the probiotic bacteria in the product remained high, while a considerable reduction in the bacterial counts was observed in the product stored at room temperature (25±1°C) and thus can be of great help in the development of probiotic food (Dhewa, Pant and Mishra, 2014).

This study demonstrates that synbiotic powder prepared by the combination of strain *L. plantarum* P10 and inulin prebiotic together with skim milk showed a relatively high stability during storage at refrigerated and room temperatures. This indicated that skim milk is a suitable cryoprotectant for freeze-dried synbiotic products.

5.4.3 Anti-foodborne bacteria of synbiotic powder

The synbiotic powder combination with *L. plantarum* P10 and inulin plus skim milk was evaluated for anti-foodborne bacteria. Antimicrobial activity of the synbiotics was investigated using cell-free culture supernatants (CFCS). The synbiotic powder was stored at refrigerated and room temperature for 90 days, the results showed that the antibacterial activity against 4 pathogenic bacteria of the synbiotic powder keeping at refrigerated temperature was remained until 12 weeks of storage time. Whereas, there was no inhibitory activity of the synbiotic powder that was kept at room temperature for 12 weeks.

Antimicrobial activity of synbiotic was reported in previous research of *Lactobacillus* strain. The combination of *L. fermentum* 907 with fructooligosaccharide (scFOS) and *B. longum* 1011 with isomalto-oligosaccharide (IMO) could be potential synbiotics with antimicrobial activity against both *E. coli* O157:H7 and *E. coli* O86 *in vitro*. A short chain scFOS and an IMO proved to be the most effective substrates, enhancing antimicrobial activity for *L. fermentum* and *B. Longum*, respectively (Likotrafiti et al., 2013).

5.4.4 Survival rate of synbiotic under simulated gastrointestinal tract conditions

In the gastrointestinal tract, LAB strains must first tolerate the upper gastrointestinal tract environment. The pH of the stomach is generally 2.5–3.5, forming an effective barrier against the entry of external bacteria (Huang and Adams, 2004). This study shows the survival of synbiotic under simulated gastrointestinal conditions,

resistance to amylase enzyme, gastric juice, and intestinal juice. Viability of probiotic in the synbiotic powder under simulated GIT of the synbitotic during storage at refrigerated temperature was from 11.84±0.01 to 7.82±0.05 Log CFU/mL and the cell viability of probiotic those storaged at room temperature decreased from 10.77±0.03 to 7.72± 0.02 Log CFU/mL.

Some studies reported that the survival rate of *L. fermentum* R6 at 3 h (97.0%) was not significantly different from that at 1 h (98.0%) ($p > 0.05$). The survival of *L. brevis* R4 and *L. plantarum* in simulated gastric juice decreased significantly at 3 h, these rates were still over 90%, so they were considered gastric acid-tolerant strains. The survival rate of *L. curvatus* R5 was significantly lower than those of the other nine strains, with only 63.7% and 61.4% survival at 1 and 3 h, respectively. The acid resistance of LAB strains is dependent on the specific strain and species. Besides, the lower pH in fermented environments contributes to the acid tolerance of LAB strains. Although *L. curvatus* R5 and *L. curvatus*, and *L. fermentum* R6 and *L. fermentum* are the same species, their survival rates (3 h) showed significant differences ($P < 0.05$). Meanwhile, the H+-ATPase activity of the LAB also impacted the acid tolerance of LAB strains (Leite et al., 2015). The data reported by Han et al., that determined the survival of LAB incubated in simulated intestinal juice after incubation in the simulated gastric juice (pH 8) showing that all 10 LAB strains survived in the simulated intestinal juice. The survival rates of the tested strains decreased with incubation time but were all still over 84% at the end of the incubation period. *L. brevis* R4, *L. curvatus* R5, *L. curvatus*, and *L. pentosaceus* showed greater ability to tolerate intestinal juice, and their survival rates exceeded 90% at 8 h. Although *L. curvatus* R5 was sensitive to the simulated gastric juice, it showed greater viability in the simulated intestinal juice (Han et al., 2017). Resistance to simulated gastric fluids pH adjusted to 2.5 for 3 h simulated intestinal fluid pH 8 for 3 h of the probiotic strains: *Lactobacillus helveticus* and *Lactobacillus plantarum* were found strongly resistant to various conditions. *Lactobacillus rhamnosus, L. lactis, P. acidilactici,* and *S. thermophilus* were resistant to the artificial gastric juice but were inhibited to a greater extent by simulated intestinal fluid. *P. acidilactici* was the least sensitive among the latter strains, while the two strains of *Lactobacillus acidophilus, Lactobacillus buchneri* and *S. thermophilus* where much less resistant. *L. farciminis* had very poor resistance properties in both gastric and

intestinal conditions. (Grimoud et al., 2010). Exposure to gastric and intestinal fluids along the digestive tract is the main stress that could decrease the viability of ingested probiotics.

Most of the strains were resistant to gastric conditions but had more variable resistance profiles to the artificial intestinal fluid. The decreasing in viability after the transition to intestinal fluid from gastric fluid has been described previously. It may be due to the rapid pH shift from acidic to basic conditions. The observed variations of such resistance could be explained by strain dependent responses, as reported previously (Grimoud et al., 2010). The pH in the human stomach ranges from 1, during fasting, to 4.5, after a meal, and food ingestion can take up to 3 h. Since *Lactobacillus* strains are known to survive at pH 4.6, lower pH values were examined. Although all the examined strains were completely resistant to pH 3 even after 4 h of exposure, most of the strains displayed loss of viability when exposed to pH 1 for 1 h. These results are in agreement with those obtained from similar studies, where *Lactobacillus* strains were able to retain their viability when exposed to pH values of 2.5–4.0, but displayed loss of viability at lower pH values (Conway, Gorbach and Goldin, 1987). Conway et al. have been studied the combined effect of a pepsin-pH solution simulating the gastric juice. The result was not clear whether the decrease of viability conferred by the pepsin solution at pH 2 was due to the enzyme alone or in synergy with low acidity. It should be mentioned that probiotic bacteria are mainly consumed in the presence of milk proteins. Milk proteins have a protective effect on the starters and thus support bacterial survival in the acidic environment of the stomach (Fernández, Boris and Barbes, 2003). Also, the gastric juice itself may offer some degree of protection, when compared with low pH buffers. In contrast to pepsin, most strains examined in this study could survive well in a pancreatin solution at pH 8.0 or in the presence of bile salts (0.3%, w/v), simulating the near-neutral small intestine environment (Conway, Gorbach and Goldin, 1987).

The research showed the general decrease in survival over the storage time by all probiotics, and our results agreed with those reports. Source of sugars from inulin that can be metabolized and reduction in ATP have been indicated as factors that affect survival rates of synbiotic bacteria in acidic environments (Corcoran et al., 2005). The addition of prebiotics like inulin has been reported to protect probiotics against stress in acidic environments. Prebiotics like inulin achieves the protective role of probiotics

against gastric juice and bile salts by binding water in the food, forming a gel made up of a tridimensional network of microcrystals that interact forming small aggregates (Buriti, Castro and Saad, 2010). Studies involving the effect of storage time and food matrices on probiotic viability and survival are important in the development of most efficient synbiotic products.

The study by Dodoo et al. (2017) of the commercial products contained probiotic strains (freeze-dried *L. acidophilus* LA5) indicated that all the commercial probiotics and the prepared freeze-dried strain demonstrated over 10^6 CFU/ml reductions within 5 min. When these were encapsulated for site-specific delivery into the distal parts of the gut, viabilities of approximately 90% were obtained after these capsules had been initially deposited in gastric acid for 2 h (Dodoo et al., 2017).

In the study by Jang et al. (2019), the viability of the encapsulated cells was assessed under various stress conditions that are common to the processing and ingestion of probiotics, such as freeze-drying, exposure to simulated gastric juice (SGJ), and exposure to bile salt. During freeze-drying to make powder, *L. plantarum* levels decreased by 1.50 log colony forming units (CFU)/ml without encapsulation. When encapsulated with 0.5% γ-PGA400 under the same conditions, *L. plantarum* levels decreased by 0.19 log CFU/ml. In the SGJ condition (pH 2), all *L. plantarum* bacteria died within 1 h without encapsulation but exhibited the highest viability (decrease of 0.30 log CFU/ml) when encapsulated with 0.5% γ-PGA400. (Jang et al., 2019).

5.5 Conclusion

Traditional Thai fermented foods is a good sources of probiotic LAB. This study successfully isolated and identified LAB isolates from fermented foods that demonstrated potential probiotic properties. The strain *L. plantarum* P10 isolated from fermented fish (Pla-Som) was the candidate probiotic strain that confer basic requirement characteristics of probiotic. This strain is able to inhibit pathogenic bacteria with the ability of acid and bile salt tolerance. An adherence to Caco-2 cell and safety test of the strain *L. plantarum* P10 were also examined. The results obtained from this study indicated that *L. plantarum* P10 satisfied the property criteria for potential probiotics and may be suitable for applying in many food matrices since it possesses no

hemolytic and DNase activities. In addition, the co-culture assay showed the greatest inhibitory activity of *L. plantatrum* P10 against *S. aureus*.

Moreover, freeze dried synbiotic powder containing *L. plantarum* P10 demonstrated survivability under the simulated gastrointestinal (GI) conditions. However, the synbiotic powder formulated with skim milk showed the higher GI tolerance than the synbiotic powder containing maltodextrin. The freezed dried synbiotic powder formulated with skim milk showed the good stability in both cell viability and antibacterial activity during the storage at refrigerated and room temperature for 12 weeks. In addition, the antibacterial activity against 4 pathogenic bacteria of the synbiotic powder keeping at refrigerated temperature was remained until 12 weeks of storage time. Whereas, there was no inhibitory activity of the synbiotic powder that was kept at room temperature for 12 weeks. The obtained results from this study indicates that the strain *L. plantarum* P10 satisfies the criteria as a potential probiotic to be used as effective probiotic and suitable for application in synbiotic product providing anti-food borne pathogens.

5.6 Suggestions

L. plantarum P10 is preferable as this LAB strain has been regarded as Generally Recognized As Safe (GRAS) and has also been associated with a variety of fermented food products. According to the results obtained from this study performing by *in-vitro* studies, the effects of interactions between the host, food, and bacteria cannot be demonstrated accurately. The further investigation by *in vivo* studies are necessary to fully validate its safety and beneficial roles to the human hosts.

The application of *L. plantarum* P10 as probiotic culture in the other food or health products is also interesting to examined.

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APPENDICES

APPENDIX A
CULTURE MEDIA

CULTURE MEDIA

1. de Man, Rogosa and Sharpe (MRS) broth

MRS	55.15	g
Distilled water	1,000	mL

Dissolve 55.15 g of the powder in 1 liter of distilled water. Mix thoroughly and warm gently until solution is complete. Sterilize by autoclaving at pressure 15 Ib/in^2 , 121 °C for 15 minutes.

2. de Man, Rogosa and Sharpe (MRS) agar

MRS agar	67.15	g
Distilled water	1,000	mL

Dissolve 67.15 g of the powder in 1 liter of distilled water. Mix thoroughly and heat with frequent agitation and boil to completely dissolve the powder. Sterilize by autoclaving at pressure 15 Ib/in^2, 121 °C for 15 minutes.

3. Tryptic Soy (TSB) Broth

TSB	30	g
Distilled water	1,000	mL

Dissolve 30 g of the powder in 1 liter of distilled water. Mix thoroughly and warm gently until solution is complete. Sterilize by autoclaving at pressure 15 Ib/in^2, 121 °C for 15 minutes.

4. Tryptic Soy (TSA) agar

TSA	40	g
Distilled water	1,000	mL

Dissolve 40 g of the powder in 1 liter of distilled water. Mix thoroughly and heat with frequent agitation and boil to completely dissolve the powder. Sterilize by autoclaving at pressure Ib/in^2, 121 °C for 15 minutes.

5. Deoxyribonuclease (DNase) agar

DNase agar	42	g
Distilled water	1,000	mL

Dissolve 42 g of the powder in 1 liter of distilled water. Mix thoroughly and heat with frequent agitation and boil to completely dissolve the powder. Sterilize by autoclaving at pressure 15 Ib/in^2, 121 °C for 15 minutes.

6. Mueller Hinton (MHA) agar

MHA	38	g
Distilled water	1,000	mL

Dissolve 38 g of the powder in 1 liter of distilled water. Mix thoroughly and heat with frequent agitation and boil to completely dissolve the powder. Sterilize by autoclaving at pressure 15 Ib/in^2, 121 °C for 15 minutes.

7. Mannitol salt agar (MSA)

MSA	111.02	g
Distilled water	1,000	mL

MSA contains a high concentration of salt, making it selective for *Staphylococci* (and *Micrococcaceae*) since this level of NaCl is inhibitory to most other bacteria. It is also a differential medium, containing mannitol and the indicator phenol red. Coagulase-positive *Staphylococi* produce yellow colonies with yellow zones, whereas coagulase-negative *Staphylococi* produce small pink or red colonies with no color change to the medium.

Dissolve 111.02 g of the powder in 1 liter of distilled water. Mix thoroughly and heat with frequent agitation and boil to completely dissolve the powder. Sterilize by autoclaving at pressure 15 Ib/in^2, 121 °C for 15 minutes.

8. MacConkey ager

MacConkey ager	42	g
Distilled water	1,000	mL

It is a culture medium designed to grow Gram-negative bacteria and stain them for lactose fermentation. It contains bile salts, crystal violet dye, neutral red dye, lactose and peptone.

Dissolve 42 g of the powder in 1 liter of distilled water. Mix thoroughly and heat with frequent agitation and boil to completely dissolve the powder. Sterilize by autoclaving at pressure 15 Ib/in^2, 121 °C for 15 minutes.

APPENDIX B
REAGENTS

REAGENTS

1. 0.85% Normal saline

NaCl	0.85	g
Distilled water	100	mL

Dissolve 0.85 g of the powder in 100 mL of distilled water. Mix thoroughly until solution is complete. Sterilize by autoclaving at pressure 15 Ib/in^2 , 121°C for 15 minutes.

2. Phosphate – buffer saline (PBS)

NaCl	9	g
$Na_2HPO_4.2H_2O$	9	g
KH_2PO4	1.5	g
3 M HCl	adjust pH	
3 M NaOH		
Distilled water	1,000	mL

Dissolve all chemical in 1000 mL of distilled water. Mix thoroughly until solution is complete. The final pH is 2.0, 3.0 and 6.5 with 3 M HCl and 3 M NaOH. Sterilize by autoclaving at pressure 15 Ib/in^2, 121 °C for 15 minutes.

3. Bile salt

Ox bile (oxgall) powder	0.15 and 0.3	g
MRS broth	55	g
Distilled water	1,000	mL

Dissolve all chemical in 1000 mL of distilled water. Mix thoroughly until solution is complete. The final concentration of bile salt is 0.15% and 0.3%. Sterilize by autoclaving at pressure 15 Ib/in^2, 121°C for 15 minutes.

APPENDIX C
DATA SUPPLEMENTS

Table C.1 83 LAB isolated from various fermented foods that correlated with isolate number

No. of product groups	Isolate number	Foods	Local name	Sources	Date of sample collection
1	KJ 1 KJ 2 KJ 3 KJ 5	Pickled kimchi	Kimchi-Pak-Gard-Dong	Tesco Lotus Supermarket, Warin Chamrap District, Ubon Ratchathani Province.	9 February 2016
2	FC 2	Pickled crab	Puu-Dong	Warin Charoensri Market, Warin Chamrap District, Ubon Ratchathani Province.	8 February 2016
3	FFC 1 FFC 2 FFC 5 FFC 6 FFC 7	Fermented minced fish	Pla-Som-Fug	Tesco Lotus Supermarket, Warin Chamrap District, Ubon Ratchathani Province.	9 February 2016
4	FFPR 1 FFPR 2 FFPR 3 FFPR 4 FFPR 5 FFPR 6 FFPR 7 FFPR 8	Pickled fish	Plaa-Raa	Warin Charoensri Market, Warin Chamrap District, Ubon Ratchathani Province.	8 February 2016
5	FFPS 1 FFPS 2 FFPS 3 FFPS 4 FFPS 5 FFPS 6	Pickled fish	Pla-Som (Pla-Sa-Wai)	Warin Charoensri Market, Warin Chamrap District, Ubon Ratchathani Province.	8 February 2016
6	MR 1 MR 2 MR 3 MR 4 MR 5	Mushroom nham	Nham-Hed	Tesco Lotus Supermarket, Warin Chamrap District, Ubon Ratchathani Province.	9 February 2016
7	PSS 1 PSS 2 PSS 3 PSS 5	Pork and Rice Sausage	Sai-Krok-Isan	Warin Chamrap Market, Warin Chamrap District, Ubon Ratchathani Province.	9 February 2016

Table C.1 83 LAB isolated from various fermented foods that correlated with isolate number (Continued)

No. of product groups	Isolate number	Foods	Local name	Sources	Date of sample collection
8	P01 P02 P03 P04 P05	Thai sour pork	Nham-Moo	Warin Charoensri Market, Warin Chamrap District, Ubon Ratchathani Province.	11 April 2016
9	P06 P07 P08 P09 P10	Pickled fish	Pla-Som (Pla Nuanchan)	Warin Charoensri Market, Warin Chamrap District, Ubon Ratchathani Province.	11 April 2016
10	P11 P13 P14 P15	Pickled fish	Pla-Som-Yasothorn	Night Market, Yasothorn District, Yasothorn Province.	11 April 2016
11	P19 P20	Pickled fish	Pla-Som (Pla Soi)	Warin Charoensri Market, Warin Chamrap District, Ubon Ratchathani Province.	11 April 2016
12	P21 P22 P23 P25	Preserved fish roe	Som-Khai-Pla	Warin Charoensri Market, Warin Chamrap District, Ubon Ratchathani Province.	11 April 2016
13	P26 P27 P28 P29 P30	Preserved fish roe	Som-Kee-Pla (Sai Pla)	Warin Charoensri Market, Warin Chamrap District, Ubon Ratchathani Province.	11 April 2016
14	P33 P34 P35	Thai traditional fermented beef sausage	Mum-Wua	Warin Charoensri Market, Warin Chamrap District, Ubon Ratchathani Province.	11 April 2016
15	P56 P57 P59	Pickled fish	Pla-Som-Yasothorn	Ubon Ratchathani Market, Ubon Ratchathani District, Ubon Ratchathani Province.	23 April 2016

Table C.1 83 LAB isolated from various fermented foods that correlated with isolate number (Continued)

No. of product groups	Isolate number	Foods	Local name	Sources	Date of sample collection
16	P61 P62 P63 P64 P65	Pickled fish	Pla-Som (Pla Soi)	Ubon Ratchathani Market, Ubon Ratchathani District, Ubon Ratchathani Province	23 April 2016
17	P67 P69 P70	Preserved fish roe	Som-Khai-Pla	Ubon Ratchathani Market, Ubon Ratchathani District, Ubon Ratchathani Province	23 April 2016
18	P71 P72 P73 P74 P75	Thai traditional fermented beef sausage	Mum-Nuer	Ubon Ratchathani Market, Ubon Ratchathani District, Ubon Ratchathani Province	23 April 2016
19	P76	Pork sausage	Sai-Krok-Moo	Tesco Lotus Supermarket, Ubon Ratchathani District, Ubon Ratchathani Province	20 June,2016
20	P77 P78 P79	Fermented beef	Nham-Wua	Phibun Mangsahan Municipal Food Market, Phibun Mangsahan District, Ubon Ratchathani Province	20 June,2016
21	P80 P81	Fermented pork	Nham-Moo	Phibun Mangsahan Municipal Food Market, Phibun Mangsahan District, Ubon Ratchathani Province	20 June,2016

Table C.2 Antimicrobial activity of 83 LAB isolates

No.	Isolates	Antimicrobial activityInhibition zone (mm.) (mean± SD)					
		E. coli	***P. aeruginosa***	***S. enteritidis***	***S. typhimurium***	***B. cereus***	***S. aureus***
1	KJ 1	12.33±2.08	15.00±0.00	8.33±0.58	10.00±1.73	9.67±1.15	16.00 ±1.00
2	KJ 2	12.67±0.58	12.00±2.00	11.67±0.58	10.67±2.08	8.67±0.58	15.67±0.58
3	KJ 3	12.00±1.41	12.00±2.00	8.33±0.58	8.00±0.00	8.67±1.15	13.67±0.58
4	KJ 5	13.67±2.08	13.33±1.15	10.33±2.08	9.33±2.31	10.00±0.00	15.33±0.58
5	FC 2	11.00±2.00	13.67±2.08	11.67±1.15	11.67±2.08	-	17.00±1.73
6	FFC 1	-	-	8.00±0.00	-	-	-
7	FFC 2	9.33±1.53	10.00±1.00	8.33±0.58	-	8.00±0.00	11.00±2.00
8	FFC 5	10.33±1.15	13.33±1.15	8.67±1.15	8.67±1.15	9.00±1.73	13.00±2.00
9	FFC 6	8.00±0.00	8.00±0.00	8.00±0.00	-	9.33±0.58	10.00±1.00
10	FFC 7	8.67±1.15	-	8.00±0.00	-	-	8.00±0.00
11	FFPR 1	8.00±0.00	8.33±0.58	8.00±0.00	8.67±1.15	8.33±0.58	13.00±1.41
12	FFPR 2	11.67±0.58	15.00±1.41	12.00±1.00	15.00±1.41	10.67±0.58	21.00±1.41
13	FFPR 3	11.33±1.53	12.00±1.41	9.67±1.53	8.00±0.00	10.00±1.73	16.00±0.00
14	FFPR 4	8.00±0.00	8.33±0.58	8.00±0.00	8.00±0.00	9.33±1.53	12.50±2.12
15	FFPR 5	9.00±1.73	9.33±1.53	8.00±0.00	8.33±0.58	8.00±0.00	9.00±1.73
16	FFPR 6	8.67±1.15	8.33±0.58	8.00±0.00	-	8.00±0.00	13.00±1.00
17	FFPR 7	8.00±0.00	8.33±0.58	8.00±0.00	-	8.33±0.58	12.00±1.73
18	FFPR 8	8.00±0.00	14.33±1.15	8.00±0.00	10.67±0.58	8.33±0.58	8.00±0.00
19	FFPS 1	11.33±1.53	14.67±1.53	12.67±0.58	12.00±1.73	-	21.00±1.00
20	FFPS 2	10.33±1.53	13.67±1.53	11.67±1.53	10.00±1.73	-	17.67±1.15
21	FFPS 3	11.00±1.41	15.00±2.00	11.00±1.73	12.33±2.08	-	17.33±1.15
22	FFPS 4	-	-	12.33±0.58	-	-	-
23	FFPS 5	8.00±0.00	8.00±0.00	8.00±0.00	9.33±2.31	8.67±1.15	11.33±0.58
24	FFPS 6	8.00±0.00	8.33±0.58	8.00±0.00	-	9.67±2.08	13.00±1.00

Table C.2 Antimicrobial activity of 83 LAB isolates (Continued)

No.	Isolates	Antimicrobial activity Inhibition zone (mm.) (mean± SD)					
		E. coli	***P. aeruginosa***	***S. enteritidis***	***S. typhimurium***	***B. cereus***	***S. aureus***
25	MR 1	9.00±1.00	14.33±1.15	9.67±1.15	11.00±0.00	-	12.67±2.08
26	MR 2	8.00±0.00	10.67±1.53	-	14.67±0.58	-	9.67±2.08
27	MR 3	12.00±0.00	11.00±0.58	9.33±1.53	8.67±1.15	8.67±1.15	13.00±1.73
28	MR 4	8.00±0.00	11.33±0.58	8.67±1.15	8.33±0.58	8.00±0.00	10.33±2.08
29	MR 5	-	-	-	-	-	-
30	PSS 1	9.67±2.08	11.00±1.00	9.67±1.53	13.67±0.58	8.00±0.00	8.67±1.15
31	PSS 2	9.33±1.15	12.33±0.58	8.00±0.00	12.33±0.58	9.00±1.73	8.50±0.71
32	PSS 3	11.33±1.53	9.67±1.53	8.00±0.00	11.67±0.58	8.00±0.00	9.00±1.00
33	PSS 5	8.67±1.15	8.00±0.00	8.00±0.00	-	9.33±2.31	10.67±2.08
34	P01	16.33±1.53	18.00±1.00	17.00±1.41	15.50±0.71	14.00±1.73	13.33±1.53
35	P02	14.67±1.15	18.00±1.00	17.33±0.58	11.00±0.00	16.00±1.41	15.67±1.15
36	P03	15.67±0.58	15.50±0.71	16.33±1.15	17.33±0.58	13.00±1.00	13.00±1.00
37	P04	14.67±1.53	16.67±1.53	17.00±0.00	11.50±0.71	15.50±0.71	14.33±1.53
38	P05	15.67±0.58	14.00±0.00	16.00±1.41	11.00±0.00	17.33±0.58	14.00±1.73
39	P06	16.33±1.15	15.33±0.58	16.67±1.53	11.33±1.53	12.50±0.71	21.00±1.00
40	P07	15.67±0.58	15.67±0.58	16.50±0.71	12.00±1.73	16.00±2.00	13.33±1.53
41	P08	16.33±1.15	15.33±0.58	16.33±1.15	12.00±1.41	13.00±0.00	16.00±2.00
42	P09	16.33±0.58	16.33±1.53	19.00±1.00	9.67±0.58	14.50±0.71	13.00±0.00
43	P10	18.00±1.00	16.00±1.00	19.00±1.00	15.00±0.00	15.50±0.71	14.50±0.71
44	P11	16.33±0.58	17.00±1.41	16.50±2.12	11.50±0.71	18.00±1.00	11.67±0.58
45	P13	9.67±0.58	11.33±0.58	8.33±0.58	9.67±0.58	8.00±0.00	8.67±0.58
46	P14	8.67±0.58	13.33±1.53	8.67±0.58	11.67±0.58	9.67±1.15	9.67±1.15
47	P15	8.33±0.58	12.00±1.00	8.00±0.00	10.67±2.52	10.33±1.53	9.33±1.53
48	P19	10.00±1.00	12.00±0.00	8.00±0.00	13.00±1.41	10.33±1.15	8.67±0.58

Table C.2 Antimicrobial activity of 83 LAB isolates (Continued)

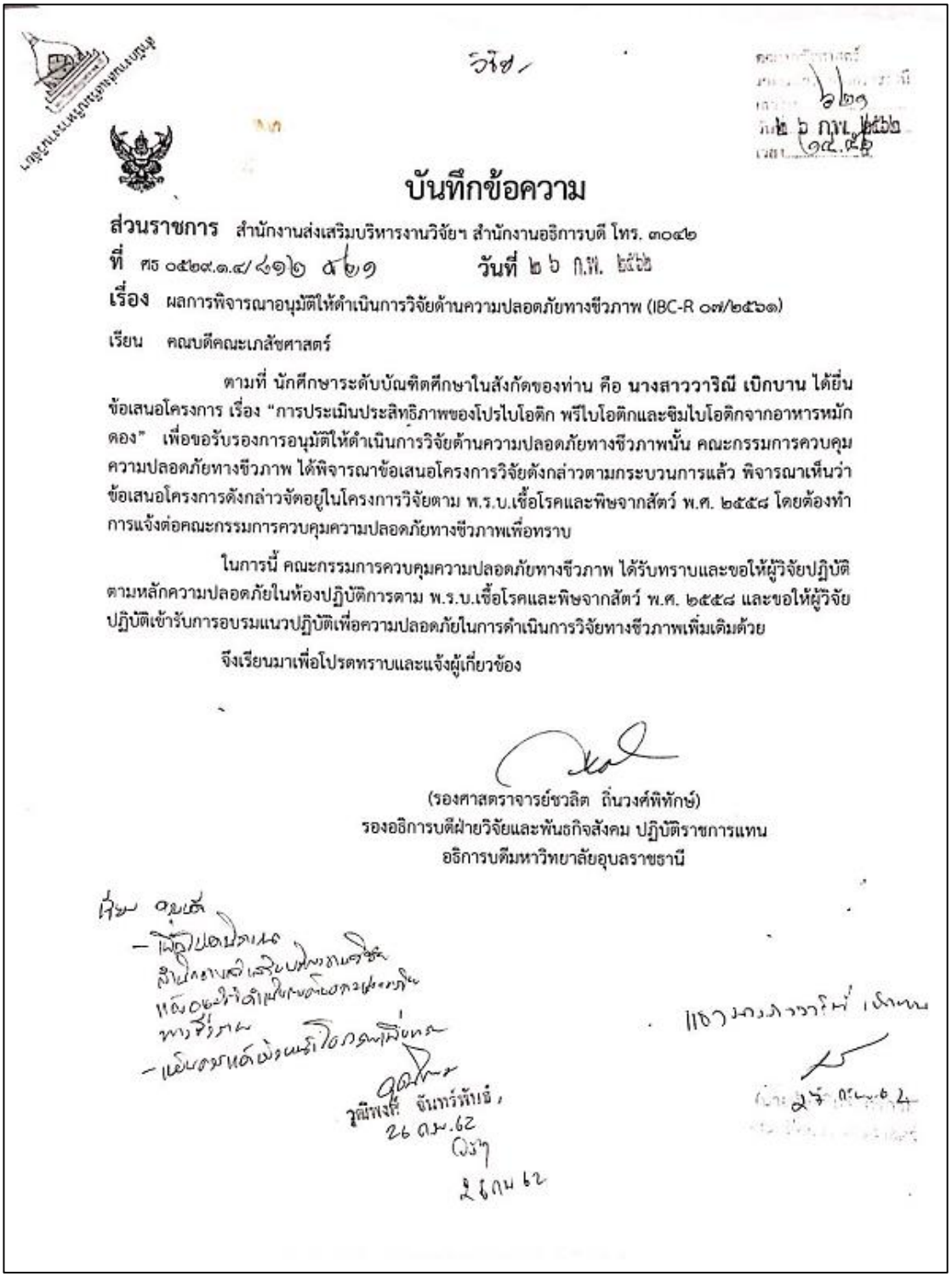
No.	Isolates	Antimicrobial activity Inhibition zone (mm.) (mean± SD)					
		E. coli	***P. aeruginosa***	***S. enteritidis***	***S. typhimurium***	***B. cereus***	***S. aureus***
49	P20	9.67±1.53	12.00±1.00	8.67±0.58	11.33±0.58	8.67±1.15	8.33±0.58
50	P21	8.67±0.58	11.33±1.53	9.33±2.31	10.33±1.53	8.33±0.58	8.00±0.00
51	P22	8.00±0.00	10.67±0.58	8.00±0.00	9.33±1.53	9.33±1.53	9.00±1.73
52	P23	8.00±0.00	10.67±0.58	9.00±1.73	11.00±1.00	9.00±1.00	8.33±0.58
53	P25	9.00±1.73	14.00±1.00	9.00±1.73	10.67±1.53	10.00±1.73	9.00±1.00
54	P26	8.33±0.58	10.67±1.53	8.67±0.58	11.67±1.53	10.00±1.00	10.33±0.58
55	P27	8.00±0.00	10.67±0.58	9.00±1.73	10.33±1.15	8.67±0.58	8.33±0.58
56	P28	8.33±0.58	10.00±0.00	9.67±1.53	12.00±1.00	9.33±0.58	-
57	P29	8.33±0.58	11.00±0.00	9.00±1.73	10.67±1.53	10.00±1.00	8.67±1.15
58	P30	8.67±0.58	10.67±0.58	8.67±1.15	9.67±1.53	8.67±0.58	8.00±0.00
59	P33	8.33±0.58	11.00±1.73	8.33±0.58	9.33±1.15	10.33±1.53	8.00±0.00
60	P34	8.33±0.58	12.33±0.58	8.00±0.00	11.00±2.00	9.00±1.00	8.67±1.15
61	P35	8.00±0.00	14.33±1.15	10.00±1.73	10.00±2.00	9.67±1.53	10.67±0.58
62	P56	8.67±0.58	13.00±1.00	9.33±0.58	11.67±0.58	9.67±1.53	15.00±1.00
63	P57	8.00±0.00	12.00±1.00	8.67±0.58	10.67±0.58	9.33±1.15	15.00±1.00
64	P59	8.00±0.00	12.00±1.73	8.67±0.58	10.67±0.58	10.67±0.58	15.67±1.53
65	P61	8.67±0.58	-	8.33±0.58	9.67±2.08	9.33±1.53	15.67±1.53
66	P62	9.00±1.00	-	8.00±0.00	9.00±0.00	8.33±0.58	13.67±1.53
67	P63	8.33±0.58	-	8.00±0.00	9.33±1.15	8.67±1.15	13.67±1.15
68	P64	8.33±0.58	-	8.00±0.00	9.33±1.15	9.00±1.00	13.67±1.53
69	P65	8.33±0.58	-	8.67±0.58	9.33±0.58	8.67±0.58	14.33±2.08
70	P67	8.00±0.00	12.67±2.08	9.33±0.58	9.67±0.58	8.33±0.58	14.00±1.73
71	P69	8.00±0.00	13.33±1.53	8.67±0.58	10.33±0.58	11.00±1.00	13.67±0.58
72	P70	8.00±0.00	12.50±0.71	8.67±0.58	10.00±0.00	9.33±0.58	14.67±0.58

Table C.2 Antimicrobial activity of 83 LAB isolates (Continued)

No.	Isolates	Antimicrobial activity Inhibition zone (mm.) (mean± SD)					
		E. coli	***P. aeruginosa***	***S. enteritidis***	***S. typhimurium***	***B. cereus***	***S. aureus***
73	P71	9.00±1.00	12.00±1.41	8.00±0.00	9.00±1.00	8.00±0.00	17.00±1.41
74	P72	9.33±1.53	12.00±0.00	8.00±0.00	9.50±0.71	9.00±1.00	16.00±0.00
75	P73	8.67±0.58	-	8.33±0.58	8.33±0.58	9.00±1.00	13.67±1.15
76	P74	8.67±0.58	-	8.00±0.00	8.67±0.58	9.00±1.73	14.00±0.00
77	P75	8.00±0.00	11.67±2.31	9.33±0.58	11.00±0.00	10.00±2.00	15.00±1.00
78	P76	-	-	8.67±0.58	-	8.00±0.00	-
79	P77	-	8.00±0.00	8.67±0.58	-	-	8.33±0.58
80	P78	-	8.00±0.00	8.33±0.58	-	-	8.33±0.58
81	P79	-	-	-	8.00±0.00	-	-
82	P80	8.00±0.00	8.00±0.00	-	8.67±0.58	-	8.33±0.58
83	P81	-	8.00±0.00	-	-	-	8.00±0.00
84	MRS broth	-	-	-	-	-	-
85	Oxytretracycline (50 µg/ml)	19.56±1.17	21.33±1.53	17.44±2.17	28.56±1.71	33.56±2.22	31.33±1.53

APPENDIX D

CERTIFICATE OF INSTITUTIONAL BIOSAFETY



บันทึกข้อความ

ส่วนราชการ สำนักงานส่งเสริมบริหารงานวิจัยฯ สำนักงานอธิการบดี โทร. ๓๐๔๒

ที่ ศธ ๐๕๒๙.๑.๔/๔๑๒ ๕๒๑ **วันที่** ๒๖ ก.พ. ๒๕๖๒

เรื่อง ผลการพิจารณาอนุมัติให้ดำเนินการวิจัยด้านความปลอดภัยทางชีวภาพ (IBC-R ๐๗/๒๕๖๑)

เรียน คณบดีคณะเภสัชศาสตร์

ตามที่ นักศึกษาระดับบัณฑิตศึกษาในสังกัดของท่าน คือ **นางสาววาริณี เบิกบาน** ได้ยื่นข้อเสนอโครงการ เรื่อง "การประเมินประสิทธิภาพของโปรไบโอติก พรีไบโอติกและซิมไบโอติกจากอาหารหมักดอง" เพื่อขอรับรองการอนุมัติให้ดำเนินการวิจัยด้านความปลอดภัยทางชีวภาพนั้น คณะกรรมการควบคุมความปลอดภัยทางชีวภาพ ได้พิจารณาข้อเสนอโครงการวิจัยดังกล่าวตามกระบวนการแล้ว พิจารณาเห็นว่าข้อเสนอโครงการดังกล่าวจัดอยู่ในโครงการวิจัยตาม พ.ร.บ.เชื้อโรคและพิษจากสัตว์ พ.ศ. ๒๕๕๘ โดยต้องทำการแจ้งต่อคณะกรรมการควบคุมความปลอดภัยทางชีวภาพเพื่อทราบ

ในการนี้ คณะกรรมการควบคุมความปลอดภัยทางชีวภาพ ได้รับทราบและขอให้ผู้วิจัยปฏิบัติตามหลักความปลอดภัยในห้องปฏิบัติการตาม พ.ร.บ.เชื้อโรคและพิษจากสัตว์ พ.ศ. ๒๕๕๘ และขอให้ผู้วิจัยปฏิบัติเข้ารับการอบรมแนวปฏิบัติเพื่อความปลอดภัยในการดำเนินการวิจัยทางชีวภาพเพิ่มเติมด้วย

จึงเรียนมาเพื่อโปรดทราบและแจ้งผู้เกี่ยวข้อง

(รองศาสตราจารย์ชวลิต ถิ่นวงศ์พิทักษ์)
รองอธิการบดีฝ่ายวิจัยและพันธกิจสังคม ปฏิบัติราชการแทน
อธิการบดีมหาวิทยาลัยอุบลราชธานี

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