SURVIVAL OF PROBIOTIC BACTERIA IN ICE CREAM FROM CONCENTRATED YOGURT AND KEFIR

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Finally, the author hopes that this thesis will be useful for those interested in studying about making yogurt and kefir to the development and application of yogurt and kefir into industrial.

Sasiwimon Kunthong

หัวข้อวิทยานิพนธ์ การรอดชีวิตของแบคทีเรียโพรไบโอติกในไอศกรีมจากโยเกิร์ตเข้มข้นและคีเฟอร์

ผู้เขียน นางสาวศศิวิมล ขุนทอง

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บทคัดย่อ

จุลินทรีย์โพรไบโอติกเป็นจุลินทรีย์ที่มีประโยชน์ต่อร่างกาย โดยเฉพาะในระบบทางเดินอาหาร ซึ่งจะช่วยรักษาสมดุลของลำไส้และกำจัดจุลินทรีย์อื่นที่อาจส่งผลเสียต่อร่างกาย ในการศึกษานี้มีจุดมุ่งหมายเพื่อตรวจสอบการรอดของจุลินทรีย์ที่ถูกเลือกเป็นโพรไบโอติกในไอศกรีมที่ทำจากโยเกิร์ตเข้มข้นและคีเฟอร์ระหว่างการเก็บรักษา และเพื่อพัฒนาให้ได้ผลิตภัณฑ์ดังกล่าวที่มีแบคทีเรียโพรไบโอติกหรือแบคทีเรียที่มีประโยชน์ในปริมาณที่เพียงพอ ในการศึกษา ได้ตรวจวิเคราะห์การรอดชีวิตของแบคทีเรียโพรไบโอติก 3 ชนิด ได้แก่ *Bifidobacterium bifidum* B4140, *Lactobacillus casei* subsp. *casei* B1922 และ *Leuconostoc mesenteroides* TISTR473 ในไอศกรีมจากโยเกิร์ตเข้มข้นที่มีความแตกต่างของปริมาณไขมันนมและชนิดของสารให้ความหวาน รวมถึงได้ศึกษาการรอดชีวิตของจุลินทรีย์ในไอศกรีมคีเฟอร์ ผลการศึกษาพบว่า *B. bifidum* B4140, *L. casei* B1922 และ *L. mesenteroides* TISTR473 ในผลิตภัณฑ์ไอศกรีมโยเกิร์ต มีเปอร์เซ็นต์การรอดชีวิตตลอดระยะการเก็บรักษา 90 วัน ที่ -20 °C เฉลี่ยเท่ากับ 56.15-74.77, 63.90-78.64 และ 66.62-66.76 เปอร์เซ็นต์ ตามลำดับ ซึ่งปริมาณไขมันในนมและชนิดของสารให้ความหวานไม่มีผลอย่างมีนัยสำคัญทางสถิติสำหรับการรอดชีวิตของ *L. casei* และ *L. mesenteroides* แต่สารให้ความหวานอาจมีผลต่อการรอดชีวิตของ *B. bifidum* ($p < 0.05$) โดยรวมแล้วกระบวนการผลิตไอศกรีมโดยใช้โยเกิร์ตเข้มข้นที่ทำจากนมสดหรือนมพร่องมันเนย รวมถึงสารให้ความหวานจากธรรมชาติที่รายงานในการศึกษานี้เป็นกระบวนการที่สามารถใช้ในการผลิตผลิตภัณฑ์ไอศกรีมที่ทำจากโยเกิร์ตเข้มข้นและคีเฟอร์ ซึ่งยังคงมีเชื้อแบคทีเรียโพรไบโอติกและแบคทีเรียในคีเฟอร์จำนวนไม่น้อยกว่า 6 log CFU/g หลังจาก 3 เดือนของการเก็บรักษาในสภาพแช่แข็ง

Thesis Title	Survival of Probiotic Bacteria in Ice cream from Concentrated Yogurt and Kefir
Author	Miss Sasiwimon Kunthong
Degree	Master of Science (Applied Microbiology)
Advisor	Assistant Professor Dr. Thararat Chitov

ABSTRACT

Probiotic microorganisms are beneficial to the body, especially the gastrointestinal system, for they help maintain the intestinal balance and eliminate microorganisms that may adversely affect the body. The aims of this study were to examine the survival of microorganisms that were selected as probiotics in ice cream made of concentrated yogurt and concentrated kefir and to develop these ice cream products that have sufficiently high numbers of probiotic or beneficial bacteria. Survival of probiotic bacteria, including *Bifidobacterium bifidum* B4140, *Lactobacillus casei* subsp. *casei* B1922 and *Leuconostoc mesenteroides* TISTR473, in yogurt ice cream in relation to milk fat contents and types of sweeteners, and total microorganisms in kefir ice cream were studied. The results showed that *B.bifidum* B4140, *L. casei* B1922 and *L. mesenteroides* TISTR473 in the yogurt ice cream product that was stored at -20 °C for 90 days had average survival percentages of 56.15-74.77, 63.90-78.64, and 66.62-66.76, respectively. The milk types (the fat contents in the milk) and the types of sweeteners did not seem to have a significant effect on survival of the probiotic *L. casei* and *L. mesenteroides*, but a possible effect from sweeteners on survival of *B. bifidum* was noted ($p < 0.05$). Overall, the process for production of ice cream from concentrated yogurt from whole or skimmed milk and natural sweeteners were developed. The processes supported survival of probiotics in yogurt ice cream and live bacteria in kefir, which survived at acceptably high numbers, ≥6 log CFU/g, in these products after 3 months of frozen storage.

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LIST OF ABBREVIATIONS

CFU	Colony forming unit
CFU/ml	Colony forming unit per milliliter
g	gram
h	hour
mg	milligram
min	minute
mm	millimeter
nm	nanometer
pH	Potential of Hydrogen ion
psi	Pound Per Square Inch
rpm	rounds per minute
sec	second
µl	microliter

LIST OF SYMBOLS

˚C	Degree Celsius
%	percent

CHAPTER 1

General Introduction and Thesis Outline

1.1 Introduction

At present, consumers have paid more attention to health. This can be seen by the increasing demands for foods that are beneficial to health, food supplements, and food having natural and functional ingredients. For this reason, the development of functional food products is in the trend in food product development. The functional ingredients can be chemical compositions of food, such as fibers, vitamin, minerals and antioxidants; and microbial composition, such as probiotic microorganisms.

The definition of probiotics is "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" (FAO/WHO, 2001). The benefits of probiotics that have been reported include boosting immunity, potentially preventing colon cancer, preventing inflammations, and acting against gastrointestinal disorders (Kailasapathy *et al*., 2008; Forsgård, 2019). Dairy products such as pasteurised milk, butter, yogurt, cheese, and ice cream are considered useful vehicles for probiotics (Mc Brearty *et al*., 2001; Farnworth *et al*., 2007; Isik *et al*., 2011; Shori *et al*., 2018). Nowadays, probiotic products are gaining attention for industries that target the development of functional foods (Saad *et al*., 2013), and the products can be in the forms of probiotic food or food supplement, containing microbes that are beneficial to health, especially in the digestive system (Mountzouris and Gibson, 2003; Homayouni *et al*., 2008). Probiotic microbes help to maintain the balance of gut microorganisms and eliminate microbes that may adversely affect the body, allowing the body to function well. Probiotic microorganisms can be corporated in food products or can be used as part of production or fermentation processes. Since milk is considered to have nutrients suitable for the growth of many probiotic bacteria, it is potential to be used for probiotic product development. There are

dairy products that contain various probiotic microbes, such as curd milk, yogurt, and drinking yogurt (Gharibzahedi and Chronakis, 2018).

One important requirement of probiotic food products is the sufficient amout of live probiotics in the products. Survival of probiotic cultures in probiotic products, especially those undergo heat treatment or frozen storage, such as frozen yogurt or ice cream yogurt is important (Abadía-García *et al*., 2013). The minimum level of probiotic bacteria in a probiotic product throughout the shelf life that is advised to be in the range of 10^6 - 10^9 CFU/g or ml (Shah, 2007). Characterised probiotic strains such as Lactobacilli and Bifidobacteria are widely accepted as probiotics and have been made available for human use (Toma and Pokrotnieks, 2006; Salminen *et al*., 2005). This has been an obstacle for developing new probiotic food products, because many probiotic cultures may not survive food processing conditions or food storage conditions to remain in the sufficient levels as required. In the case of ice cream or frozen dessert, they have become popular and can be a potential vehicle for probiotics (Haynes and Playne, 2002). Frozen yogurt or ice cream yogurt has an advantage over milk-based ice cream and non-fermented frozen dessert because many consumers with lactose intolerance (LI) can consume it without having adverse gastrointestinal (GI) symptoms, such as bloating, diarrhea, and abdominal pain including allergy (Miranda *et al*., 2011; Rangel *et al*., 2016; Abdelazez *et al*., 2017). Therefore, probiotic food products such as frozen yogurt which contain lactic acid bacteria that have the ability to digest lactose in milk (He *et al*., 2008) offer double benefits of carrying probiotics into the body and addressing lactose intolerance problem. Probiotic microbes are also known to reduce the risk of various diseases, thus improve intestinal health (Kechagia *et al*., 2013) and general health in turn.

This research work was proposed to study the survival of different types of probiotics in frozen yogurt in relation to fat contents of milk and types of sweeteners. The work will focus on the process of yogurt and ice cream making, the selection of probiotic strains, the survival of probiotic bacteria in the ice cream products, and the effects of fat contents and types of sweeteners on the survival of probiotic bacteria. Finally, the products were developed to functional ice cream products from probiotic yogurt and kefir which can serve as alternatives of functional food for consumers.

1.2 Objectives

1) To investigate the survival of selected probiotics in ice cream made from concentrated yogurt and kefir, in relation to the production process, fat contents, and types of sweeteners.

2) To develop functional ice cream products from concentrated yogurt and kefir that have sufficiently high numbers of probiotic or beneficial bacteria.

CHAPTER 2

Literature review

2.1 Milk and Dairy Products as Sources of Nutrients and Beneficial Microbes

Milk is a solution of various dissolved compounds produced from the mammary glands of mammals. Milk that are popular for consumption is obtained from cows and goats. However, milk from other animals such as yaks, camels, mare, ewes, is also consumed in some regions of the world. Milk is well-known as a rich source of nutrients. Many products from milk, especially fermented milk (dairy) products can also be an important source of beneficial microbes.

2.1.1 Composition of milk

Milk contains milk fat which is dispersed in water as fat globules causing oil-in-water emulsions. Another substantial part of milk is protein, especially casein, lactoalbumin and lactoglobulin, which are in the colloidal form. It also contains sugar, amino acids, vitamin and minerals, which are dissolved in a true solution or as crytalloids (Mehta, 2015). Changes in the proportions of the suspended particles of any kind will have a significant impact on physical and chemical properties of milk. The chemical composition of cow's milk contains 85.5-89.5% water, 10.5-14.5% total solid, 2.5-6.0% milk fat, 2.9-5.0% protein, 3.6-5.5% lactose and 0.6-0.9% minerals. The average composition of milk consists of 87% water, 4.0% fat, 4.8% lactose, 3.4% proteins and 0.8% minerals (Burke *et al*., 2018). Normal cow's milk should have milk solid non-fat (MSNF) of approximately 8.25% and Milk fat not less than 3.25%. However, the chemical composition of cow's milk varies depending on various factors including breeds of cows, age, feeding period, breast condition, as well as external factors such as types of feed, seasons and the environment (Varnam and Sutherland, 2001).

2.1.2 Milk and dairy products as potential sources of probiotics

Because milk is nutritious, as it contains proteins, milk fat, sugar (lactose), amino acids, vitamin and minerals (Mehta, 2015), it is a natural source of many beneficial bacteria. Many milk products, such as yogurt, kefir and kumiss are also known as potential sources of probiotics (Ershidat, and Mazahreh, 2009). Products made from these fermented milk can also be good probiotic carriers. Probiotic dairy products should have one important characteristics: they must have sufficient amounts of probiotic microorganisms to establish in the hosts' body so that they can benefit the hosts (Shi *et al*., 2016).

One of the potential probiotic products that is made of fermented milk products is probiotic ice cream yogurt (or probiotic frozen yogurt). This product has become increasingly popular because of its extended shelf-life. Probiotic ice cream can be produced by incorporation of probiotic bacteria in both fermented and unfermented mixtures. (Akin *et al*., 2007; Hekmat and McMahon, 1992; Kailasapathy and Sultana, 2003; Ravula and Shah, 1998). The benefits of probiotic bacteria in the dairy products depend on number of live bacteria, type of dairy foods, and presence of air and storage temperature (Homayouni *et al*., 2008). For the products with extended shelf-life, the viability of probiotic cultures must be maintained throughout the product's shelf-life. Therefore, International Dairy Federation (IDF) recommends that a minimum of 10^7 probiotic bacterial cells should be alive at the time of consumption per gram of a probiotic product (Homayouni *et al*., 2008). However, probiotic bacteria may not survive in sufficiently high numbers in frozen dairy products unless a suitable processing method is used (Dave and Shah, 1998). There are many factors that can affect the survival of probiotics in ice cream such as probiotic strains, osmotic pressure, packaging, pH and ice cream ingredients.

2.2 Probiotic bacteria

The word probiotic comes from Greek root, meaning "for life". The term was first used in 1965 by Lilly and Stillwell. A group of experts have defined probiotics as "Living microorganism which upon ingestion in certain numbers exert health benefits beyond

inherent general condition" (Guarner and Schaafsma, 1998). The more recent definition of probiotics are given as "distinct as live microorganisms which, when administered in sufficient amounts present a health benefit on the host" (FAO/WHO, 2002). Probiotic microorganisms must have the following properties: they must be resistant to acids and bile salt; they must have the ability to adhere to the surface of intestinal mucosa and produce anti-microbial substances to inhibit microorganisms that do not cause health benefits. Because of these properties, they can help balance intestinal microbes and create other health benefits for hosts (Bielecka, 2006). Probiotic bacteria mainly belong to the group of lactic acid bacteria including *Lactobacillus* groups such as *L. acidophilus, L. plantarium, L. reuteri, L. salivarius, L. casei, L. johnsonii* and *L. gasseri. Bifidobacterium* is another major group of bacteria known for the probiotic properties. The potential probiotic species include *Bifidobacterium bifidum, B. lactis, B. longum, B. breve, B. infantis, B. thermophilum* and *B. pseudolongum* (Makarova *et al*., 2006). The reason that make many *Lactobacillus* and *Bifidobacterium* species potential probiotics is because both bacterial genera are dominant in the intestine. *Lactobacillus* species colonise the small intestine and *Bifidobacterium* species colonise the large intestine (Walter, 2008). Apart from these groups, *Streptococcus* and *Enterococcus* are counted as potential probiotic bacteria. Moreover, there are studies that points to yeasts as probiotic microorganisms (Fijan, 2014).

Probiotic bacteria can be used in many ways to sustain or improve health and to prevent or treat various diseases. Bacteria of the *Lactobacillus* group produces enzymes β-galactosidase (Hsu *et al*., 2007), which help reduce the amount of lactose in food and therefore, reduce occurrence of diarrhea due to lactose intolerant. In addition, many substances produced by probiotics, such as organic acids, free fatty acids, ammonia, hydrogen peroxide and bacteriocins, can act as natural antibiotics and thus help eliminate harmful bacteria that co-exist in food. Probiotic bacteria also help inhibit toxins from bacteria by blocking the toxin entering the cells. They also can compete with intestinal pathogens by colonizing adhesion sites on intestinal tissues (Piatek *et al*., 2012). Moreover, they can stimulate the immune system in the intestines and blood stream or stimulate other cells to fight with pathogens and stimulate the creation of anti-disease

agents in the body such as gamma-interferon, interleukin-12, interleukin-1841 (Savan, 2006; Villena *et al*., 2008). Probiotics have been associated with a variety of health benefits, including improved digestion, better immunity, improved heart health and even increased weight loss (Ritchie and Romanuk, 2012; King *et al*., 2014).

There are many groups of bacteria known to be potential probiotics. These include lactic acid bacteria, acetic acid bacteria and bifidobacteria.

2.2.1 Lactic acid bacteria

Lactic acid bacteria (LAB) are given such name because their main end product of carbohydrate metabolism is lactic acid (Lebeer *et al*., 2008). The lactobacilli group are one of the most common LAB found in the human body (Fig. 2.1). Certain species such as *Lactobacillus acidophilus* and *Lactobacillus casei* are parts of the normal flora of humans. They are found in the oral cavity, the small intestine and the vaginal epithelium, where they are thought to play beneficial roles.

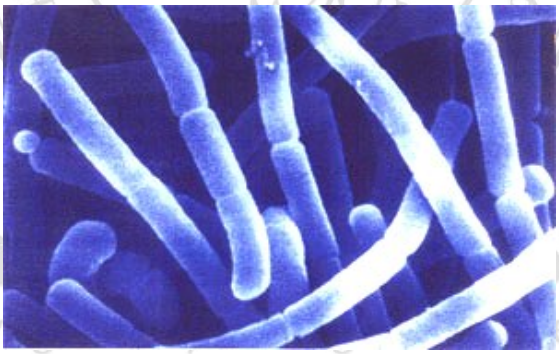

Figure 2.1 *Lactobacillus* sp. (Todar, 2019)

Lactic acid bacteria have diverse metabolic pathways, some of which clearly contribute to the flavours, tastes and textures of the food products in which they are present. The following are some examples of etabolisms.

1) Degradation of citric and sorbic acid

Various lactic acid bacteria can cause decomposition of citric acid (Figure 2.2) to generate a range of products, principally lactic acid, acetic acid, and other products such as acetoin and 2,3-butanediol (Moreno and Peinado, 2012).

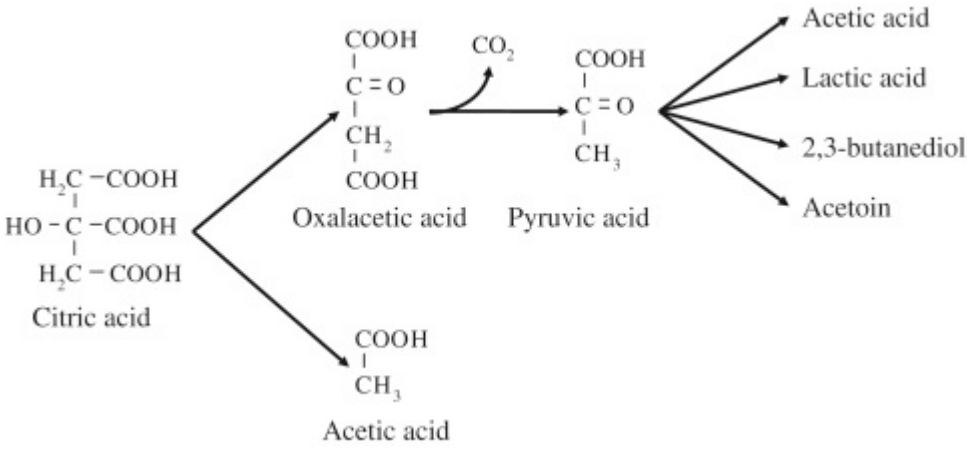

Figure 2.2 Degradation of citric acid (in Enological Chemistry, 2012)

2) Degradation of sorbic acid

Lactic acid bacteria such as *Leuconostoc oenos* and Heterolactic acid bacteria of the genus *Lactobacillus* (of the species *brevis* and *hilgardii*) are responsible for this transformation (Figure 2.3). One of the compounds responsible for the aroma of geraniums is 2-ethoxy-3,5-hexadiene, which is a powerful odorant (Moreno and Peinado, 2012).

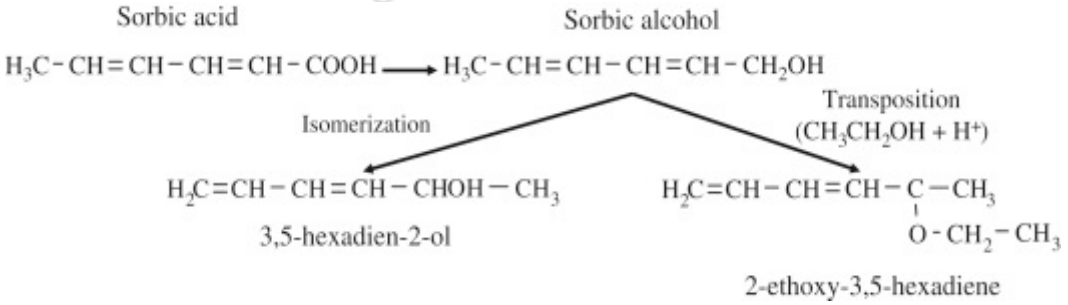

Figure 2.3 Degradation of sorbic acid (in Enological Chemistry, 2012)

2.2.2 Acetic bacteria

Acetic acid bacteria are obligate aerobes. However, a number of acetic acid bacteria was able to grow despite the anaerobic conditions present during alcoholic fermentation which was not favorable for their growth (Guillamón and Mas, 2017).

Acetic acid bacteria (AAB) are capable of oxidising ethanol as a substrate to produce acetic acid in neutral and acidic media under aerobic conditions. They are Gram-negative, acidophilic α-proteobacteria and are widespread in nature. The above characteristics make them involved in the production of fermented foods such as chocolate products, coffee, vinegar and beers. These characteristics, however, can be detrimental and make them causes of spoilage of beers, wines and ciders. *Acetobacter* and *Gluconobacter* are the two main genera in AAB for acetic acid fermentations. Members of the genus *Acetobacter* were historically differentiated from those of the genus *Gluconobacter* by a preference for ethanol and the ability to overoxidise acetate to CO_2, usually when ethanol is depleted (Xu *et al*., 2011).

2.2.3 Bifidobacteria

Bifidobacteria is Gram-positive, Y-shape bacteria that grow in anaerobic condition. This group of bacteria are generally helpful in maintaining appropriate balances between the various flora in different sections of the human intestine, making them potential probiotics. Probiotics such as *B. bifidum* have become very popular lately because it has been shown to have the ability to treat some diseases such as necrotising enterocolitis, a type of infection in the intestinal lining caused by harmful irritable bowel syndrome (IBS) and to treat certain kinds of diarrhea (Duggal, 2017). Furthermore, they can enhance lactose digestion for some people with lactose intolerant condition, colonise the intestinal tract, prevent or help improve acute diarrhea caused by foodborne infection and prevent antibiotic-induced diarrhea (Robinson, 2014). Some strains of *Bifidobacterium* are capable of synthesising certain vitamin, for example, thiamine, folic acid, biotin, and nicotinic acid (Rossi *et al*., 2011).

2.3 Yogurt and concentrated yogurt

2.3.1 History of concentrated yogurt

Yogurt is a dairy product that are widely consumed throughout the world. It has a semi-solid consistency and made from milk mixed with the starter culture consisting of *Streptococcus thermophiles* and *Lactobacillus delbrueckii* subsp. *bulgaricus* (Kefir, 2014). Concentrated yogurt, the type of yogurt that has thick, viscous texture because the liquid is drained out, is often being marketed in North America under the name "Greek yogurt". Yogurt production and consumption are similar in the Levant region, Eastern Mediterranean, Middle East, Central Asia and South Asia (Fisberg and Machado, 2015). Concentrated yogurt is often used as a cooking ingredient because this type of yogurt contains enough fat to prevent the protein in yogurt from forming at high temperatures.

Concentrated or Greek-style yogurt has more protein and fat content than ordinary yogurt (Moore *et al.*, 2018; Chandan *et al.*, 2017). The amount of carbohydrates is 50% less than the typical yogurt because the amount of sugar is extracted along with the whey to make the Greek yogurt concentrated. Therefore, it has less effect on those who are allergic to lactose sugar than general yogurt. Greek yogurt also has lower sodium than yogurt and is suitable for those who want to control weight and reduces risks of high blood pressure and heart disease (Castaneda and Haupt, 2018).

2.4 Kefir

2.4.1 History of kefir

Kefir dates back many centuries to the shepherds of the Caucasus mountains. They discovered that fresh milk carried in leather pouches would occasionally ferment into an effervescent beverage. Kefir was scarcely known outside the Caucasian Mountains, although Marco Polo mentioned it in recounting his travels (Oerman and Libudzisz, 2012). Kefir continues to be popular in Russia, southwestern Asia, Eastern and Northern Europe, and has recently gained some popularity in the United States (Baschali *et al.*, 2017).

Kefir is made by fermentation of the "kefir" grains, which resembles miniature cauliflowers that are the size of wheat kernels (Mueller, 2014). These grains consist of casein and gelatinous colonies of microorganisms that are grown together symbiotically. The dominant yeasts in kefir are *Kluyveromyces marxianus*, *Kluyveromyces laetis* and *Saccharomyces cerevisiae*. Other species encountered were *Saccharomyces unisporus*, *Saccharomyces rouxii*, *Torulaspora delbrueckii* and *Debaryomyces hansenii*. As for bacteria, the main groups found in kefir included *Lactobacillus caucasicus*, *Leuconnostoc* sp., lactic streptococci and acetic acid bacteria (Loretan, 1999; Tamime, 2002; Singh *et al.,* 2018). Because of the unique combination of fermenting microorganisms, it results in the product being a thick and tangy beverage. Kefir is known to contain health-promoting bacteria (Chen *et al*., 2015). It contains calcium, amino acids, B-vitamin and folic acid (Sanders *et al*., 2007). Studies have shown that kefir may come with many benefits, affecting digestion, inflammation and bone health. In one study, kefir was shown to improve the digestion of lactose in 15 people with lactose intolerance (Rosa *et al*, 2017). Those who are lactose-intolerant were unable to digest the sugars in dairy products, resulting in symptoms like cramps, bloating and diarrhea (Hertzler and Clancy, 2003). Another study found that consuming 6.7 ounces (200 ml) of kefir daily for six weeks decreased markers of inflammation, which linked to the reduced risks of chronic diseases like heart disease and cancer (Adiloğlu *et al*., 2013). The cultures' chemical changes make the milk more digestible, allowing the body to absorb more of the naturally present nutrients. The transformation of lactose to lactic acid allows people, even those with lactose-intolerance, to digest kefir and get its full benefits (John and Deeseenthum, 2000). In one study, the effects of kefir on 40 people with osteoporosis (characterized by weak, porous bones) were observed. After six months, the group consuming kefir was found to have improved bone mineral density a condition (Tu *et al*., 2015), which is believed to be related to its high calcium contents (Ilesanmi-Oyelere and Kruger, 2020).

2.5 Yogurt and kefir as frozen products

Frozen yogurt or frozen fermented dairy dessert are relatively new products which can be prepared in the way similar to ice cream. Frozen yogurt has been used as a vehicle

for incorporation of probiotic bacteria such as *Lactobacillus acidophilus*, and *Bifidabaterium* spp. (Shah and Ravula, 2001). Frozen yogurt or yogurt-based ice cream is a product obtained from emulsions of fats and proteins along with other components such as sugar and frozen according to Notification of the Ministry of Public Health (No. 354), 2013. Likewise, frozen kefir or kefir-based ice cream is a mixture of kefir and other ingredients, which is processed through freezing. The ingredients often used in these types of products include sweetener(s), stabiliser and emulsifier that are added to improve physical characteristics. The products require a lactic acid content of not less than 0.3 to 0.5 % (Syed *et al*., 2018).

CHAPTER 3

Materials and Methods

3.1 Raw Materials

3.1.1 Pasteurized milk (whole milk and skimmed milk)

3.1.2 Whipping cream

3.1.3 Longan honey

3.1.4 Brown sugar

3.1.5 Yogurt

3.1.6 Kefir starters

3.2 Tools and Equipment

3.2.1 Blender (Philips, Cucina HR 1841)

3.2.2 Ice cream machine (NEMOX, Italy)

3.2.3 Texture analyser (TA.HDplusC, USA)

3.2.4 Fat Extraction System Soxhlet, (ST 243 FOSS, Denmark)

3.2.5 Moisture Analyzer (MX-50, AND, Japan)

3.2.6 Analytical Balance 2 digits (BJ 2200C, Precisa, Switzerland)

3.2.7 Analytical Balance 4 digits (XB 220A, Precisa, Switzerland)

3.2.8 Analytical Balance 4 digits (Explorer, OHAUS, USA)

3.2.9 Hot air oven (FD 56, Binder, Germany)

3.2.10 Hot air oven (E 15, Binder, Germany)

3.2.11 Spectrophotometer (Helios Epsilon, USA)

3.2.12 PCR Thermal Cycler (Eppendorf AG, Germany)

3.2.13 Autoclave (Tomy, SX-700)

3.2.14 Centrifuge (Eppendorf Centrifuge 5430R, Germany)

3.2.15 Vortex mixer (VM-300, Gemmy Industrial Corp., Taiwan)

3.2.16 pH meter (Starter 3100, OHAUS, USA)

3.2.17 Water bath (Julabo Eco Temp, TW20)

3.2.18 Ultra Slim LED Illuminator (Hercuvan)

3.2.19 Biohazard Safety Cabinets class ll (ESCO, SC2-A41)

3.2.20 Fume Hood (iim I-LAB)

3.2.21 Shaking incubator (Scientific Co., Ltd, USA)

3.2.22 Micro Sterilizer (Hercuvan, 2017)

3.2.23 Stomacher machine (Seward Stomacher 400, England)

3.2.24 Microwave (Sharp)

3.2.25 Compound light microscope (Olympus, CX1, Japan)

3.2.26 Stereo Microscope (Olympus, TL3, Taiwan)

3.2.27 Invert Microscope (Olympus, CKX41, Japan)

3.2.28 Gel electrophoresis apparatus

3.2.29 Kitchen scale 1 kg

3.2.30 Gas stove

3.2.31 Stainless pot

3.2.32 Refrigerator 4 °C

3.2.33 Refrigerator -20 °C

3.2.34 Thermometer

3.2.35 Incubator 30, 32 and 37 °C

3.2.36 Incubator 37 °C, 5% CO_2

3.2.37 Micropipette sizes 1-10 µl, 20-200, 100-1,000, 1,000-10,000 µl

3.2.38 Brix refractometer

3.2.39 Stainless Beaker (2,000 ml)

3.2.40 McFarland standards

3.2.41 Falcon cell culture flask

3.2.42 6-well plates

3.2.43 96-well plates

3.2.44 Slides and coverslips

3.2.45 Elastic bands

3.2.46 Stainless steel spoons

3.2.47 Straining cloth

3.2.48 Plastic boxes

3.2.49 Stainless dipper

3.2.50 Gloves

3.2.51 Wide mouth Duran bottles (500 and 1,000 ml)

3.2.52 Cylinders (100, 500 and 1,000 ml)

3.2.53 Beakers (50 and 250 ml)

3.2.54 Erlenmeyer flask (250 ml)

3.2.55 Lighter

3.2.56 Stomacher bags

3.2.57 Tube racks

3.2.58 Test tubes (16 × 150 mm) with lids

3.2.59 Droppers

3.2.60 Loops

3.2.61 Needles

3.2.62 Tip 10 µl, 200, 1,000 and 10 ml

3.2.63 Centrifuge tubes

3.2.64 Cuvettes

3.2.65 Micro centrifuge tubes (1.5 ml)

3.2.66 Package

3.2.67 Spreader

3.2.68 Plastic plates

3.2.69 Glass bottles

3.2.70 Weighting boats

3.2.71 Spoons

3.2.72 Plastic bags

3.2.73 Duran bottle (250, 500, 1,000 ml)

3.2.74 PCR tubes

3.2.75 Anaerobic jar

3.2.76 Gas pack (Biomérieux)

3.2.77 Bunsen burner

3.2.78 Syringe Filter (pore size 0.22 µm)

3.2.79 Syringe 10 ml

3.2.80 Neubauer hematocytometer chamber

3.2.81 Forceps

3.2.82 Desiccator

3.2.83 Thimbles

3.2.84 Thimbles stand

3.2.85 Thimbles adapters

3.2.86 Cups

3.2.87 Cups stand

3.2.88 Cups holder

3.2.89 Extraction cups

3.2.90 Glassware beaker 250 ml

3.2.91 Whatman paper No.1

3.3 Chemical and Media

3.3.1 Crystal violet

3.3.2 Iodine solution

3.3.3 Decolorizer

3.3.4 Safranin O

3.3.5 HCl

3.3.6 Cysteine

3.3.7 KOH solution

3.3.8 3% (w/v) Hydrogen peroxide solution

3.3.9 Save view (Neogreen, Korea)

3.3.10 Agarose gel (Vivantis, USA)

3.3.11 Glycerol

3.3.12 Bile salt

3.3.13 Acetic acid

3.3.14 0.1% peptone water

3.3.15 1X PBS buffer

3.3.16 Dulbecco's Modified Eagle Medium (DMEM)

3.3.17 10% fetal bovine serum

3.3.18 0.25% Trypsin-EDTA solution

3.3.19 Penicillin-Streptomycin (Pen-Strep)

3.3.20 Agar

3.3.21 Giemsa

3.3.22 Yeast extract (Difco, USA)

3.3.23 Meat extract (Difco, USA)

3.3.24 Potato extract (Difco, USA)

3.3.25 Beef extract (Difco, USA)

3.3.26 Magnesium sulphate · $7H_2O$ ($MgSO_4 \cdot 7H_2O$)

3.3.27 Manganese sulphate · $4H_2O$ ($MnSO_4 \cdot 4H_2O$)

3.3.28 HS agar and broth

3.3.29 Bromocresol purple

3.3.30 95% (v/v) Ethyl alcohol

3.3.31 Distilled water

3.3.32 Petroleum ether

3.3.33 DNA for bacteria kit

3.3.34 DNA for kefir gran kit

3.3.35 DNA for yeast kit

3.3.36 70% (v/v) Ethyl alcohol

3.3.37 Citric acid

3.3.38 Deionize water (DI water)

3.3.39 Na_2HPO_4

3.3.40 Glucose

3.3.41 Dextrose

3.3.42 Tween 80

3.3.43 Di-potassium hydrogen phosphate

3.3.44 Sodium acetate

3.3.45 Tri-ammonium citrate

3.3.46 Bromophenol blue

3.3.47 TE buffer

3.3.48 Lysis buffer

3.3.49 Chloroform

3.3.50 2x MyTaq Mix (Bioline, USA)

3.3.51 Forward primer NL1 (99822421 N. Rodrassamee 228334330 NL1)

3.3.52 Reverse primer NL4 (99822422 N. Rodrassamee 228334329 NL4)

3.3.53 Forward primer 27F (Macrogen, Korea)

3.3.54 Reverse primer 1492R (Macrogen, Korea)

3.3.55 1% agarose gel (Merck, Germany)

3.3.56 Loading dye concentration 6 times (Vivantis, Malaysia)

3.3.57 Loading dye concentration 5 times (Vivantis, Malaysia)

3.3.58 VC 1 kb DNA Ladder (Vivantis, Malaysia)

3.3.59 1X TAE Buffer

3.3.60 1% Triton X-100

3.3.61 0.85% NaCl

3.3.62 0.9% NaCl

3.3.63 Antibiotic

3.3.64 99.9% Methanol

3.3.65 Gentamycin

3.3.66 Dimethyl sulfoxide (DMSO)

3.3.67 3, [4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT)

3.4 Data Processing Program

3.4.1 SPSS statistic software version 17.0 (SPSS Inc., Chicago, Illinois, USA)

3.4.2 BioEdit Sequence Alignment Editor

3.4.3 MEGA X

3.5 Sample for Study

3.5.1 Fermented beverages (59 samples)

3.5.2 Fermented foods (30 samples)

3.5.3 Kefir samples (3 samples)

3.6 Research Methods

3.6.1 Isolation of Probiotic Microorganisms from Natural Sources and Selection of Probiotic Strains from Reference Culture Collections

Probiotics were isolated from natural sources such as fermented foods, fermented beverages, and kefir products. Fifty-four fermented beverage samples, thirty-eight fermented food samples from local markets in Chiang Mai province, and nine samples of kefir from Russia were collected. Fermented samples (10 g of each sample) were using and mixed in 90 ml of 0.1% peptone water using Stomacher. For fermented beverages 10 ml of each sample were transferred to 90 ml of 0.1% peptone water. Isolation was performed using spread plating on MRS-Cys-BPB agar (Ding and Shah, 2007). The agar plates containing isolated bacterial cultures were incubated at 37 ºC for 48 h under the anaerobic condition. Bacterial isolates were purified and tested for their morphology and biochemical reactions, such as Gram-reaction, cell morphology, and catalase reaction. Probiotic cultures were also selected from culture collections. The morphological and biochemical characteristics of the reference strains were also examined.

Probiotics strains used in this study included *Bifidobacterium bifidum* B4140, *Lactobacillus casei* subsp. *casei* B1922 (obtained from NRRL Culture Collection, USA), and *Leuconostoc mesenteroides* TISTR473 (obtained from TISTR Culture Collection, Thailand). The freeze-dried probiotic cultures from the culture collections were prepared as frozen glycerol stock cultures.

3.6.2 Preliminary Test for Probiotic Properties

The probiotic properties were primarily tested for acid tolerance and bile tolerance. Colonies that appeared on the MRS-Cys-BPB agar were transferred to MRS-Cys broth as used enrichment medium and incubated at 37 °C for 48 h. The bacterial culture were prepared to the concentration of approximately 10^8 CFU/ml (OD_{600} nm is approximately 0.1-0.2). A portion of each bacterial culture was tested for acid tolerance in MRS broth, pH 2.0 adjusted by HCl (Chung *et al*., 1999; Ding and Shah, 2007), and bile tolerance was tested using MRS broth with 0.4% bile salt (Nguyen *et al*., 2007). All of the cultures were incubated at 37 °C for 3 h. Cells that survived acidic and bile-containing conditions

were enumerated on MRS-Cys-BPB agar and calculated into log CFU/ml. Finally, the potential probiotic isolates were tested for adhesion to Caco-2 cells.

3.6.3 Identification of Microorganisms

Potential probiotic yeast isolates were identified using *26S rRNA* gene sequencing and probiotic bacteria were identified using *16S rRNA* gene sequencing.

1) DNA extraction from yeast isolates

1.1) A yeast colony on YPD agar was enriched in 3 ml YPD broth at 30 °C on a shaker incubator at 160 rpm for 18-24 h. Then, the culture was centrifuged at 8,000 rpm for 5 min.

1.2) The cell pellet was thawed using 200 µl lysis buffer and kept at -80 °C until the cells were frozen. The pellet was immersed in water at 95 °C for 1 min. This step was repeated for 3 times.

1.3) The cells were stirred (using vortex mixer) for 30 sec, 200 µl of chloroform was added and mixed for 2 min. Then, the cell suspension was centrifuged at 10,000 rpm for 5 min at room temperature.

1.4) The supernatant was transferred to the new tube that had 400 µl of ethanol and set aside for 5 min. This was then centrifuged at 10,000 rpm for 10 min at 4 °C.

1.5) The DNA pellet was washed with 500 µl of 70% ethanol and centrifuged at 5,000 rpm for 30 sec at 4 °C.

1.6) The supernatant was removed and the DNA pellet was dried at room temperature. The DNA pellet was dissolved with 20 µl TE buffer and examined using agarose gel electrophoresis.

2) Polymerase chain reaction

Yeast DNA extracted in 1) was used as a template for increasing the nucleotide sequence of D1/D2 domain large subunit ribosomal DNA or 26S rRNA

using two universal primers: forward primer NL1 (5'-GCATATCMIMGCGGAGGAA MG-3') and reverse primer NL4 (5'-GGTCCGTGTTTCMGACGG-3'), using components and PCR conditions as follows:

Compositions

2x MyTaq Mix	12.5	µl
Forward primer NL1	2	µl
Reverse primer NL4	2	µl
DNA template	1	µl
Distilled water	7.5	µl
Total volume	25	µl

Procedures

Initial denaturation	95 °C	2	min	
Denaturation	95 °C	15	sec	35 cycles
Annealing	50 °C	15	sec	
Extension	72 °C	15	sec	
Final extension	72 °C	7	min	
Cold storage	4 °C	(optional)		

The PCR products were examined using agarose gel electrophoresis. The PCR products were then purified using GF-1 Nucleic Acid Extraction Kit (Vivantis).

3) Nucleotide sequence analysis of D1/D2

The PCR products obtained from 2) were analysed for their sequences (U2 Bio Thailand sequencing service). Then, the results of nucleotide sequence analysis at D1/D2 domain were compared with the data base in NCBI (National Center for Biotechnology Information) to identify yeast types using the Basic Local Alignment

Search Tool (BLAST) program on the NCBI homepage.

4) DNA extraction from bacterial isolates

4.1) A colony of bacterial isolate grown on MRS-Cys-BPB agar was enriched in 5 ml of MRS-Cys broth, and incubated at 37 °C for 48 h under an anaerobic condition.

4.2) The enriched cultures were transferred to a microtube and centrifuged at 7,000 rpm for 10 min.

4.3) The cell pellet cells were added with extraction buffer (600 µl) and glass beads, mixed by vortexing 3 min, and 300 µl phenol was added.

4.4) The mixture was centrifuged at 15,000 rpm for 5 min (repeated 2 times), and the supernatant was transferred to a new tube.

4.5) The sample was with 30 µl of 3 M CH_3COONa, 300 µl of isopropanol, and 300 µl of absolute ethanol and mixed. It was incubated at -20 °C for 15 min.

4.6) After that, the sample was centrifuged at 15,000 rpm for 20 min.

4.7) The precipitated DNA was washed with 500 µl of 70% ethanol and centrifuged at 15,000 rpm for 10 min (repeated 2 times).

4.8) The DNA was dried overnight and dissolved with 50 µl distilled water.

5) Polymerase chain reaction

Bacterial DNA samples extracted in 4) were used as a templates in PCR identification. The universal primers: 27F (5'-AGAGTTTGATCMTGGCTCAG-3') was used as a forward primer and 1492R (5'-GGTTACCTTGTTACGACTT-3') was used as a reverse primer. The PCR reactions were as follow:

Compositions

2x MyTaq Mix	12.5	µl
Forward primer NL1	2	µl
Reverse primer NL4	2	µl
DNA template	1	µl
Distilled water	7.5	µl
Total volume	25	µl

Procedures

Initial denaturation	95 °C	5	min	
Denaturation	94 °C	45	sec	35 cycles
Annealing	56 °C	45	sec	
Extension	72 °C	2	min	
Final extension	72 °C	10	min	
Cold storage	4 °C	(optional)		

3.6.4 Selection of Probiotic Strain and Development of Probiotic Ice cream Products

1) Preparation of probiotic cultures for yogurt and kefir

For making the yogurt, a 50 µl-portion of each probiotic culture from the glycerol stock was inoculated into 5 ml deMann, Rogasa and Sharpe medium supplemented with 0.05% cysteine (MRS-Cys), pH 6.5, and incubated at 37 °C for 48 h under an anaerobic condition. Then, these were subcultured into 200 ml MRS-Cys broth, and incubated under the same condition. Cells of the probiotic cultures were harvested by centrifugation at 7,000 rpm for 10 min at 4 °C. The cell pellets were washed twice with 0.85% NaCl and centrifuged at 7,000 rpm for another 20 min. After the supernatant

was discarded, sterile distilled water was added to resuspend the cells before use. Enumeration of *B. bifidum*, *L. casei*, and *L. mesenteroides* in these inoculum preparations was carried out on MRS-Cys-BPB agar (deMann, Rogasa and Sharpe, 0.05% cysteine, 0.002% bromophenol blue). These inoculum preparations were used for yogurt production. Moreover, a commercial starter culture including *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*, *Leuconostoc mesenteroides* subsp. *cremoris*, *Kluyveromyces marxianus* were used as a starter culture for made kefir.

2) Yogurt and concentrated yogurt production process

Pasteurised whole milk (ca. 3.5% fat) and skimmed milk (0% fat) were warmed to 32-35 °C. Yogurt starters containing *L. bugaricus* and *S. thermophilus* was added to the pasteurised milk of different fat contents, as above. The pasteurised milk samples mixed with starter cultures were then divided into 3 portions, in which 3 probiotic cultures from the inoculum preparations, as above, were added to the milk at the final concentration of 1% (v/v). The mixtures were then incubated at 45 °C in a water bath until the pH values of approximately 3.8 to 4.5 were reached (taking approximately 6 h). The yogurt samples obtained were then filtered through cheesecloth at 4 °C for another 12 h to obtain a concentrated (Greek-style) yogurt.

3) Kefir and concentrated kefir production process

Starter culture from 1) was added to pasteurised whole milk (ca. 3.5% fat). The pasteurised milk samples mixed with the kefir starter was then divided into 2 portions, in which 2 types of sweeteners were added. The mixtures were then incubated at 32 °C in a water bath until the pH values of approximately 3.8 to 4.5 were reached (taking approximately 20 h). The kefir obtained was then filtered through cheesecloth at 4 °C for another 12 h to obtain concentrated kefir.

4) Production of probiotic frozen yogurt and kefir

Each of the probiotic frozen yogurt (Table 3.1) or kefir (Table 3.2) of different formulae was prepared as follows: 500 g concentrated yogurt or kefir, 71.5 g sweetener (cane sugar syrup or longan honey; for syrup, 1 part of unrefined cane sugar was mixed with 1 part of water, by weight), and 89 g whipping cream. The mixture of

each formula was mixed in a blender and pours into an ice cream maker which was operated until the desired texture of the ice cream was obtained. The ice cream products were stored at -20 °C for 90 days, a period determined based on a reasonable shelf-life of the products.

Table 3.1 The formula for ice cream yogurt

formula	**probiotic**	**milk type**	**sweetener**
1	*B. bifidum*	whole milk	cane syrup
2	*B. bifidum*	whole milk	honey
3	*B. bifidum*	skimmed milk	cane syrup
4	*B. bifidum*	skimmed milk	honey
5	*L. casei*	whole milk	cane syrup
6	*L. casei*	whole milk	honey
7	*L. casei*	skimmed milk	cane syrup
8	*L. casei*	skimmed milk	honey
9	*L. mesenteroides*	whole milk	cane syrup
10	*L. mesenteroides*	whole milk	honey
11	*L. mesenteroides*	skimmed milk	cane syrup
12	*L. mesenteroides*	skimmed milk	honey

Table 3.2 The formula for ice cream kefir

Formula	Probiotic	Milk type	Sweetener
1	Mixed kefir culture	whole	honey
2		whole	cane syrup

* whole: whole milk

3.6.5 Survival of Probiotic Microorganisms in Ice cream Products

The samples of probiotic frozen yogurt of different formulae were analysed for the number of probiotic bacteria after the mixture was prepared and stored for 30, 60 and 90 days at -20 °C. For the samples of frozen kefir, they were analysed for the total number of microorganisms. A 10 g-portion of each sample (each formula) was serially diluted in 90 ml of 0.1% (w/v) peptone water, and plated (using drop plating technique) on MRS-Cys-BPB agar (Cysteine was included at 0.05% (w/v) concentration to promote the growth of *B. bifidum* (Xing *et al.,* 2016) and bromophenol blue was added (0.002% (w/v) as a pH indicator. The agar plates were incubated at 37 °C for 48 h under an anaerobic condition (generated using the BBL gas pack). The specific colonies of each species were counted under a Stereomicroscope and calculated as CFU/g of the frozen product.

The survival of total bacteria from kefir in kefir ice cream products stored at -20 ºC were examined on day 0, 30, 60, and 90. The count was analysed on MRS-Cys-BPB agar incubated at 37 ºC for 48 h under an anaerobic condition.

3.6.6 Statistical Analyses

The data analyses were performed using SPSS statistical software version 17.0 (SPSS Inc., Chicago, Illinois, US). Microbial viability was analyzed using One Way ANOVA based on a completely randomized design (CRD) for analysis. The formulas of frozen that were added with each of probiotic culture were analyzed by Factorial Experiment base on randomized complete block (RCB) design. The detailed analysis was

based on Duncan for confidence. All of the analyses were considered statistically significant at a p-value of < 0.05.

CHAPTER 4

Results

4.1 Selection of Probiotic Strains for Use in Yogurt-based and Kefir-Based Ice Cream Products

4.1.1 Screening of Potential probiotic isolates from fermented foods

4.1.1.1 Isolation of probiotic bacteria and yeasts

One approach for selection of probiotics to be used in yogurt- and kefir-based ice cream products was to screen probiotics from natural sources. Samples of fermented products, 89 in total, including fermented beverages, local fermented meats, fermented vegetables and kefir, were spread on DeMan Rogosa Sharp-Cysteine-Bromophenol blue (MRS-Cys-BPB) agar and incubated at 37 °C for 48 h under an anaerobic condition to isolate lactic acid bacteria group. Acetic acid bacteria (expected to be found especially in fermented beverages and kefir) were isolated on Hestrin and Schramm (HS) agar and incubated at 30 °C for 48 h. Yeast extract peptone dextrose (YPD) agar was used to isolate yeasts and the plates were incubated at 30 °C for 48 h. After that, the bacterial colonies grown on each selective medium were examined for Gram-stain reaction, KOH reaction and the presence of catalase enzyme. Presumptive yeast colonies on YPD agar was examined microscopically. The characteristics of presumptive lactic acid bacteria were those that were Gram-positive, non-spore forming, negative in KOH string test, and catalase-negative. For the presumptive acetic acid bacteria, they were Gram-negative, non-spore forming, KOH-positive, and catalase-positive. The characteristics of each bacterial isolate are shown in Table 4.1.

The isolates were subjected to a preliminary test for their potential to be probiotics. Presumptive lactic acid bacteria retrieved from the selective media were cultured in MRS-Cys broth and incubated at 37 °C for 48 h under an anaerobic condition. Presumptive

acetic acid bacteria were cultured on HS broth and incubated at 30 °C for 48 h. Optical density at 600 nm (OD_{600}) was measured and the cultures were adjusted to have the density of approximately 10^8 CFU/ml. This culture preparation was used to test for acid tolerance (in the medium with pH of 2.0) and bile salt tolerance (in the presence of 0.4% ox gall). As a result, 38 bacterial isolates and 24 yeast isolates were found to be acid and bile salt tolerant (Table 4.2).

Table 4.1 Characteristics of microorganism from local fermented samples

Sample	Microorganism group	Gram stain	KOH test	Catalase test
Fa thalai chon	LAB	+ rod	-	-
Pumpkin	LAB	+ cocci	-	-
Cabbage imchi	LAB	+ short rod	-	-
Radish kimchi	LAB	+ short rod	-	-
Carambola	LAB	+ cocci	-	-
Butter fruit	LAB	+ rod	-	-
kefir	LAB	+ rod	-	-
	AAB	- short rod	+	+
	Y			
Pickled bean curd	LAB	+ short + cocci	-	-
	Y			
Mixed vegetable kimchi	LAB	+ short rod + cocci	-	-
	Y			
Spring Onion kimchi	LAB	+ short rod	-	-
Fermented tea leaves	LAB	+ rod + cocci	-	-
	Y			

Table 4.1 (Continued)

Sample	Microorganism group	Gram stain	KOH test	Catalase test
Pickled Kum	LAB	+ short rod		
	Y			
Pickled bamboo shoots	LAB	+ rod	-	-
Pickled Cabbage	LAB	+ short rod	-	-
Fermented meat (Nham)	LAB	+ short rod + cocci	-	-
	Y			
Fermented fish (Pla Som)	LAB	+ short rod + cocci	-	-
	Y			
Sweetened Rice	LAB	+ short rod + cocci	-	-
	Y			
Noni	AAB	- short rod	+	+
Black Ginger	AAB	- short rod	+	+
	Y			
Heart leaved moonseed	Y			
Passion fruit	Y			
Mango	Y			
Santo	Y			

*LAB: Lactic acid bacteria, AAB: Acetic acid bacteria, Y: Yeast

Table 4.2 Acid and bile tolerances of bacterial and yeast isolates

Characteristics of isolates	Isolate	
	bacteria	yeast
Tolerant to acid (pH 2.0) only	-	-
Tolerant to bile salt (0.4% ox gall) only	SK40, SK41, SK42, SK43, SK48, SK50, SK57, SK58, SK65, SK68, SK69, SK76, SK80, SK82, SK83	SK3, SK5, SK11, SK14, SK15, SK19, SK30, SK31, SK32, SK33, SK34, SK35, SK36
Tolerant to acid (pH 2.0) and bile salt (0.4% ox gall)	SK37, SK38, SK39, SK44, SK46, SK49, SK52, SK53, SK56, SK59, SK70, SK71, SK72, SK73, SK74, SK75, SK77, SK78, SK79, SK84, SK85, SK86, SK87, SK88, SK89, SK90, SK91, SK94, SK95, SK96, SK97, SK98, SK99, SK100, SK101, SK102, SK103	SK1, SK2, SK4, SK6, SK7, SK8, SK9, SK10, SK12, SK13, SK16, SK17, SK18, SK20, SK21, SK22, SK23, SK24, SK25, SK26, SK27, SK28, SK29

4.1.1.2 Identification of Microorganisms

Thirty-eight isolates of bacteria that had the ability to tolerate acid (pH 2.0) and bile salt (0.4% ox gall) were identified using *16S rRNA* gene sequencing. Almost all of the representatives of isolates from fresh and fermented foods and fermented beverages were in the genus *Lactobacillus* such as *L. plantarum* (26.32% of the isolates), *L. brevis* (18.42%) and *L. mesenteroides* (7.89%). The results are shown in Table 4.3. Moreover, 24 yeast isolates were identified using *26S rRNA* gene sequencing. Most of the yeast isolates belonged to the following groups: *Candida ethanolica* (20.83% of the isolates, recovered from fermented beverages), *Pichia manshurica* (16.67%, from

fermented tea leaves and fermented beverages), and *Wickerhamomyces anomalus* (12.5%, from sweetened rice (Kaomak). Moreover, *Kluyveromyces marxianus*, a lactose-fermenting yeast, which are often found in dairy products, was also isolated (Table 4.4).

Table 4.3 Bacterial species isolated from fermented samples

Bacteria isolate	**Number of species**	**Percentage of total (%)**
Lactobacillus plantarum	10	26.32
Lactobacillus brevis	7	18.42
Lactobacillus sp.	2	5.26
Lactobacillus collinoides	2	5.26
Lactobacillus suebicus	1	2.63
Lactobacillus koreensis	1	2.63
Lactobacillus zymae	1	2.63
Lactobacillus namurensis	1	2.63
Enterococcus faecium	2	5.26
Leuconostoc mesenteroides	3	7.89
Lactobacillus paracasei	2	5.26
Lactobacillus heilongjiangensis	1	2.63
Lactobacillus hilgardii	1	2.63
Lactobacillus crustorum	1	2.63
Lactobacillus nagelii	1	2.63
Rummeliibacillus suwonensis	2	5.26

Table 4.4 Yeast species isolated from fermented samples

Yeast isolate	Number of species	Percentage of total (%)
Candida ethanolica	5	20.83
Candida glabrata	1	4.17
Candida metapsilosis	1	4.17
Candida tropicalis	1	4.17
Kodamaea ohmeri	2	8.33
Pichia deserticola	2	8.33
Pichia fermantans	2	8.33
Pichia manshurica	4	16.67
Trichosporon asahii	1	4.17
Wickerhamomyces anomalus	3	12.5
Zygosaccharomyces rouxii	1	4.17
Kluyveromyces marxianus	1	4.17

4.1.2 Selection of reference probiotic strains

Although the results of the *16S rRNA* gene sequencing and the acid and bile tolerance showed some species that are potential probiotics, they were mostly belonging to the *Lactobacillus* group. From the identification results, one of the most potential probiotic species was *Lactobacillus paracasei*. However, because of the strict regulations concerning use of new probiotic strains in food products (Probiotic Microorganisms in Food) issued by the Ministry of Public Health (Ministry of Public Health, 2014), it was decided that the strains be added to the products should be selected from the reference (known) probiotic strains available in culture collections. Representatives of *Lactobacillus* group, which colonises small intestine; *Bifidobacterium*, which colonises large intestine; and *Leuconostoc*, which is expected to have positive contribution to the texture of the products due to its ability to produce exopolysaccharides, were selected. These included *Bifidobacterium longum* B41409, *Bifidobacterium bifidum* B4140, *Lactobacillus casei* subsp. *casei* B1922, (obtained from NRRL Culture Collection, USA), *Lactobacillus acidophilus* TISTR2365 and *Leuconostoc mesenteroides* TISTR473 (obtained from TISTR Culture Collection, Thailand).

The strains were cultured in MRS-Cys broth for 24 h at 37 °C under an anaerobic condition and growth rates were measured every 4 h for the period of 24 h. These probiotic strains were preliminary evaluated for their suitability to be incorporated in the products, for which they had to have a fast growth rate and can be cultured to reach a sufficient level. The results showed that *B. bifidum* B4140 had the highest growth rate compared with other probiotic bacteria tested, while *B. longum* could not meet these criteria because they could not easily be cultured to reach a sufficient level to be used commercially. For *Lactobacillus* group, *L. casei* and *L. acidophilus* were found to have satisfactory fast growth rates (Figure 4.1). Therefore, *L. casei* and *B. bifidum* were chosen as the representatives of *Lactobacillus* and *Bifidobacterium* groups, respectively. *Leuconostoc mesenteroides*, although did not reach as high levels in its stationary phase compared with other bacterial strains, it still reached a sufficient level and with the advantage of being an exopolysaccharide producer, it was also selected. These three selected probiotic strains were then cultured in MRS-Cys broth and the cells were collected for use in the probiotic products (Figure 4.2).

4.1.3 Selection of mixed probiotic culture from kefir samples

Besides probiotic bacteria that were to be used in the yogurt ice cream product, a mixed culture of natural probiotics from kefir were also selected for use in kefir-based ice cream. Three kefir inocula included:

Sample A – fresh kefir (origin: Russia, microbial composition: unknown)

Sample B – kefir grains (origin: water kefir sample, microbial composition: unknown)

Sample C – lyophilised kefir culture (origin: Russia, microbial composition: *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*, *Leuconostoc mesenteroides* subsp. *cremoris*, *Kluyveromyces marxianus* (yeast strain identified by DNA sequencing).

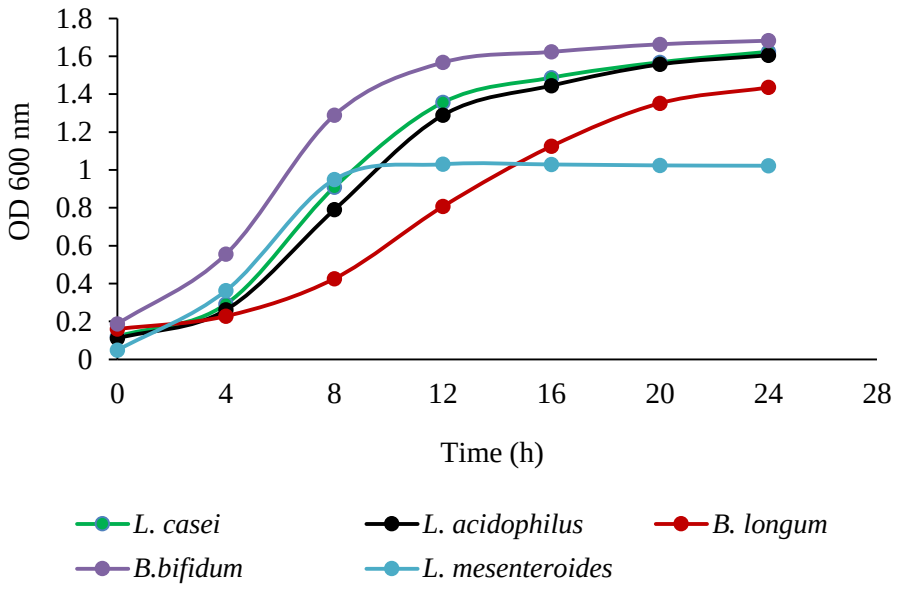

Figure 4.1 Growth rates of *B. longum* B41409, *B. bifidum* B4140, *L. casei* subsp. *casei* B1922, *L. acidophilus* TISTR2365, and *L. mesenteroides* TISTR473, as measured using OD_{600}.

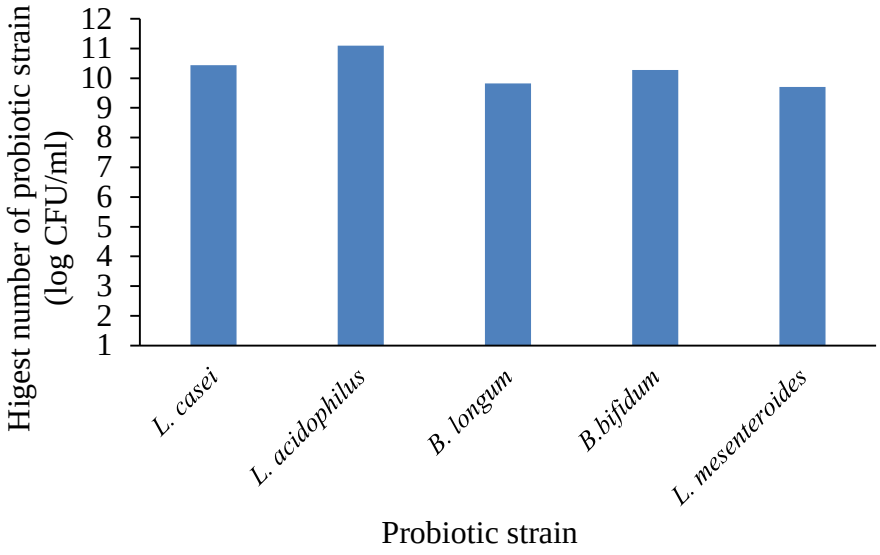

Figure 4.2 Highest numbers of *Bifidobacterium longum* B41409, *Bifidobacterium bifidum* B4140, *Lactobacillus casei* subsp. *casei* B1922, *Lactobacillus acidophilus* TISTR2365, and *Leuconostoc mesenteroides* TISTR473, after being cultured for 24 hours

As for the kefir samples, they were tested for their stability after repeated sub-culturing. Kefir made of inoculum C had the most consistent and desirable texture and flavour. This inoculum type was then selected to produce kefir for use in making kefir-based ice cream product.

4.2 Adhesion Assay for Selected Probiotic Strains

The selected probiotic strains, *Bifidobacterium bifidum* B4140, *Leuconostoc mesenteroides* TISTR473 and *Lactobacillus casei* B1922, were cultured in MRS-Cys broth at 37 °C for 48 h under an anaerobic condition. Then, the probiotic cultures were were adjusted to have OD_{600} of 0.1-0.2, using 1×PBS. The probiotic cells were harvested, washed, and DMEM + 10% FBS was added to make up to the previously adjusted concentration and the cell suspension was used as an initial inoculum for the adhesion test.

For adhesion test, the probiotic suspension (1 ml-portion of each) was dispensed on a coverslip that was coated with Caco-2 cells. The sample was then stained with Giemsa stain. The coverslips were examined under a microscope to observe cell adhesion. The surface of Caco-2 cells were adhered with probiotics, as shown in Figure 4.3. The ability of the three strains of probiotics to adhere to Caco-2 cells was expressed in percentages of cell adhesions, as shown in Table 4.5. The mixed kefir culture was subjected to the adhesion test in the same manner.

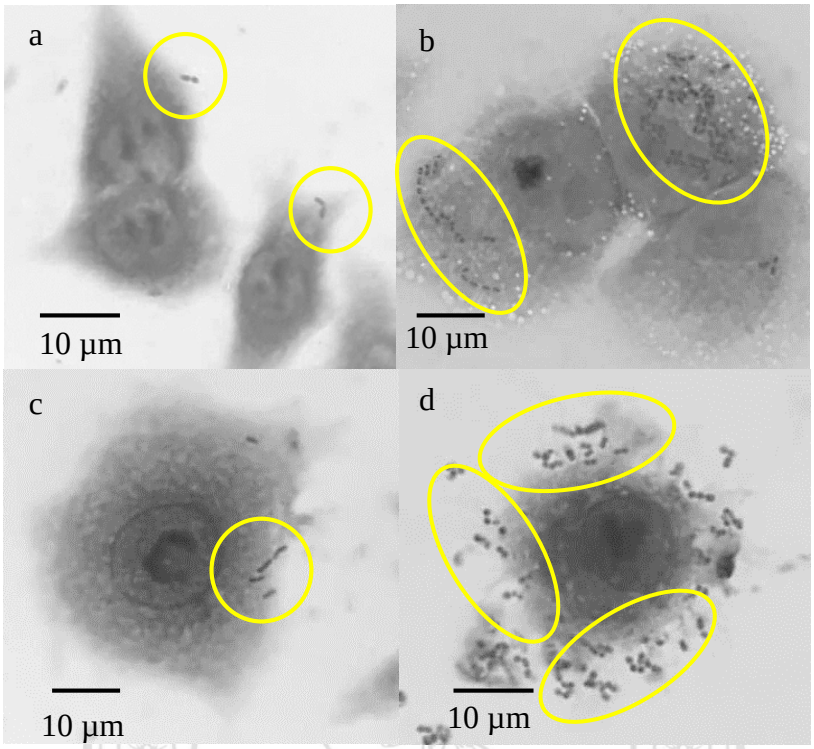

Figure 4.3 The adhesion to Caco-2 cells of probiotic strains under the microscope a) *B. bifidum* B4140, b) *L. mesenteroides* TISTR473, c) *L. casei* B1922, and d) mixed culture from kefir

Table 4.5 Percent adhesion of probiotic strains to Caco-2 cells

Probiotic strain	**Percentage of Adhesion of probiotic strain prepared at different concentrations**		
	low	**medium**	**high**
B. bifidum	42.84	49.47	58.01
L. mesenteroides	69.66	84.27	94.38
L. casei	39.43	44.05	56.26
Kefir	70.80	81.02	81.61

* L: low concentration M: medium concentration H: high concentration

4.3 Development of Yogurt Ice Cream Products

4.3.1 Preparation of probiotic inoculum for use in ice cream products

The selected probiotic bacteria were cultured in MRS-Cys broth and incubated at 37 °C for 24 h under an anaerobic condition. Then, the cells were harvested by centrifugation at 7,000 rpm for 10 min at 4 °C. The cell pellets were washed with 0.85% (w/v) NaCl, and prepared in sterile distilled water to the concentrations of 11-13 log CFU/ml (Table 4.6). These were used as fresh inoculum preparation.

Table 4.6 Concentration of cells for initial inoculum

Probiotic strains	**Initial inoculum (log CFU/ml)**
B. bifidum	12.18
L. casei	13.02
L. mesenteroides	11.17

Yogurt ice cream having each type of probiotic bacteria was prepared from yogurt made of pasteurised cow's milk with two different fat contents: whole milk and skimmed milk. The yogurt was incubated at 45 °C or until the pH reached approximately 3.8-4.0. For the kefir, it was prepared by adding the kefir starter into whole milk. The mixture was then incubated at 32 °C for approximately 18 h or until the pH dropped to 4.0-4.2 (Table 4.7). The yogurt and the kefir were then drained over a cheesecloth for 12 h. The whey recovered as a by-product from the concentrated yogurt and concentrated kefir after 12 h filtration was in the range of 50% to 65% of the original volumes.

The yogurt of different formulae were made by combining a yogurt starter containing *L. bulgaricus* and *S. thermophilus* and the probiotic culture (in the form of fresh preparations). The probiotic yogurt was further processed into concentrated (Greek-style) yogurt, which was then used as a base for the yogurt ice cream formulae. The probiotic bacteria in the whole milk yogurt reached approximately 11 log CFU/g in most samples, and their levels did not decrease when made into concentrated yogurt (Table 4.8).

Table 4.7 The pH of yogurt, concentrated yogurt, kefir, and concentrated kefir

Sample	Milk type	*B. bifidum*	*L. casei*	*L. mesenteroides*	Kefir starter
Yogurt	whole	3.89	3.85	3.93	-
	skimmed	3.78	3.78	3.86	-
Conc. yogurt	whole	3.94	3.62	3.78	-
	skimmed	3.73	3.70	3.82	-
Kefir	whole	-	-	-	4.28
Conc. kefir	whole	-	-	-	4.08

*Conc. yogurt: Concentrated yogurt, Conc. kefir: Concentrated kefir

Table 4.8 The number of probiotic bacteria and kefir starter culture in each process before making ice cream from concentrated yogurt

Sample	no. of probiotic bacteria (log CFU/g)			
	B. bifidum	*L. casei*	*L. mesenteroides*	Kefir
Yogurt	11.40	10.95	11.58	9.76
Conc. yogurt	11.12	11.90	11.48	10.66

*Conc. yogurt: Concentrated yogurt

4.3.2 Fat content analysis

The concentrated yogurt and concentrated kefir were analysed for their fat contents using the Soxtec method. Concentrated kefir shows the highest fat content, which was 8.19%. The concentrated yogurt made with *L. casei* showed lowest fat content. When the concentrated yogurt or kefir was mixed with the other ingredients in each formula and made into ice cream, the products were also analysed for their fat contents once again, since the cream or other dairy products were added as ingredients in the ice cream. The results showed that the fat contents of yogurt made with of *B. bifidum*, *L. mesenteroides* and *L. casei* were in the ranges of 7-13%, 8-10% and 8-12%, respectively, whereas the fat content of kefir was 12-13% (Table 4.9).

Table 4.9 The fat contents in concentrated samples and ice cream products

Sample	Sweetener	% Fat content			
		***B. bifidum* yogurt**	***L. mesenteroides* yogurt**	***L. casei* yogurt**	**kefir**
Conc. yogurt	-	8.15	7.39	5.29	-
Conc. kefir	-	-	-	-	8.19
Ice cream yogurt	sugar	13.26	10.31	12.56	-
	honey	7.66	8.44	8.47	-
Ice cream kefir	sugar	-	-	-	13.13
	honey	-	-	-	12.06

*Conc. yogurt: Concentrated yogurt, Conc. kefir: Concentrated kefir

4.3.3 Viscosity analysis

The viscosity of concentrated probiotic yogurt and concentrated kefir were analysed using a texture analyser. The compression and tensile forces of each sample, shown in Table 4.10 and Figure 4.4, indicated that the concentrated yogurt made with *B. bifidum* had the highest viscosity, followed by the concentrated yogurt made with *L. mesenteroides,* concentrated kefir, and concentrated yogurt made with *L. casei*, respectively.

Table 4.10 Viscosity of concentrated yogurt and kefir

Sample	Temperature (°C)	Viscosity Analysis	
		compression force	**tensile force**
Conc. *B. bifidum*	18.4	2.7258	-2.1323
Conc. *L. mesenteroides*	22.7	2.5168	-1.9311
Conc. *L. casei*	22.6	0.9423	-0.6218
Conc. Kefir	22.6	1.9467	-1.5432

*Conc.: Concentrated yogurt

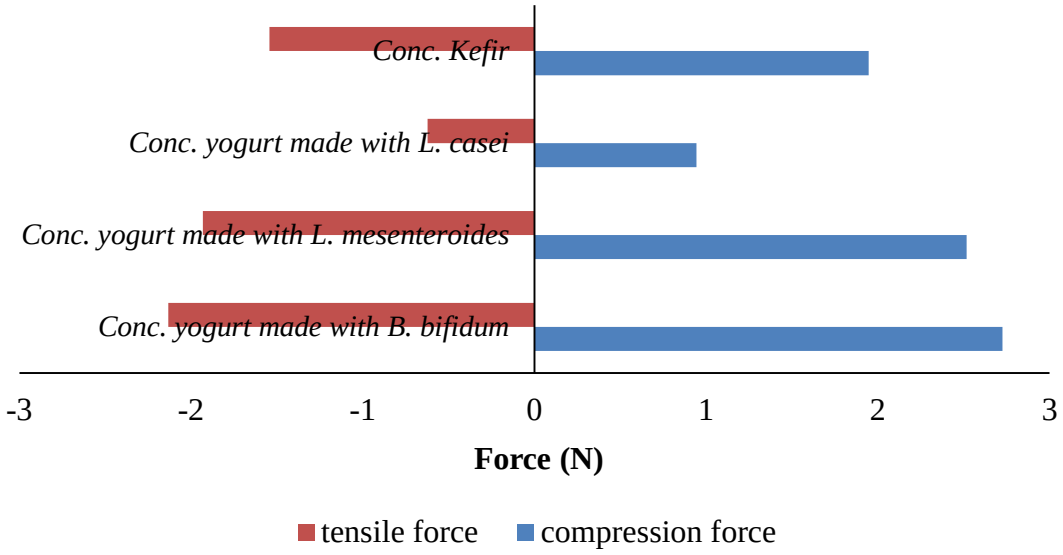

Figure 4.4 Viscosity of kefir and concentrated yogurt made with 3 probiotic strains

4.4 Survival of Probiotic Bacteria and Starter Culture in Yogurt and Kefir Ice Cream Products

The ice cream samples made with concentrated yogurts and concentrated kefir with different types of milk and sweeteners were stored at -20 °C for 90 days. Every 30 days, the yogurt ice cream samples were taken for analysis of live probiotic bacteria.

In order to evaluate how fat contents of milk affect the survival of probiotic bacteria, the percentages of survival of each probiotic bacterium in yogurt ice cream prepared from whole milk and skimmed milk with cane sugar syrup and honey were analysed. The results are shown in Figure 4.5 and Tables 4.11-4.13. The milk fat contents did not have a significant effect on survival of the three probiotic strains in both yogurt ice cream formulae (cane sugar syrup and honey formulae) in general. A significant difference in probiotic survival relating to the milk fat contents was observed only in the honey formula of yogurt ice cream made with *B. bifidum.*

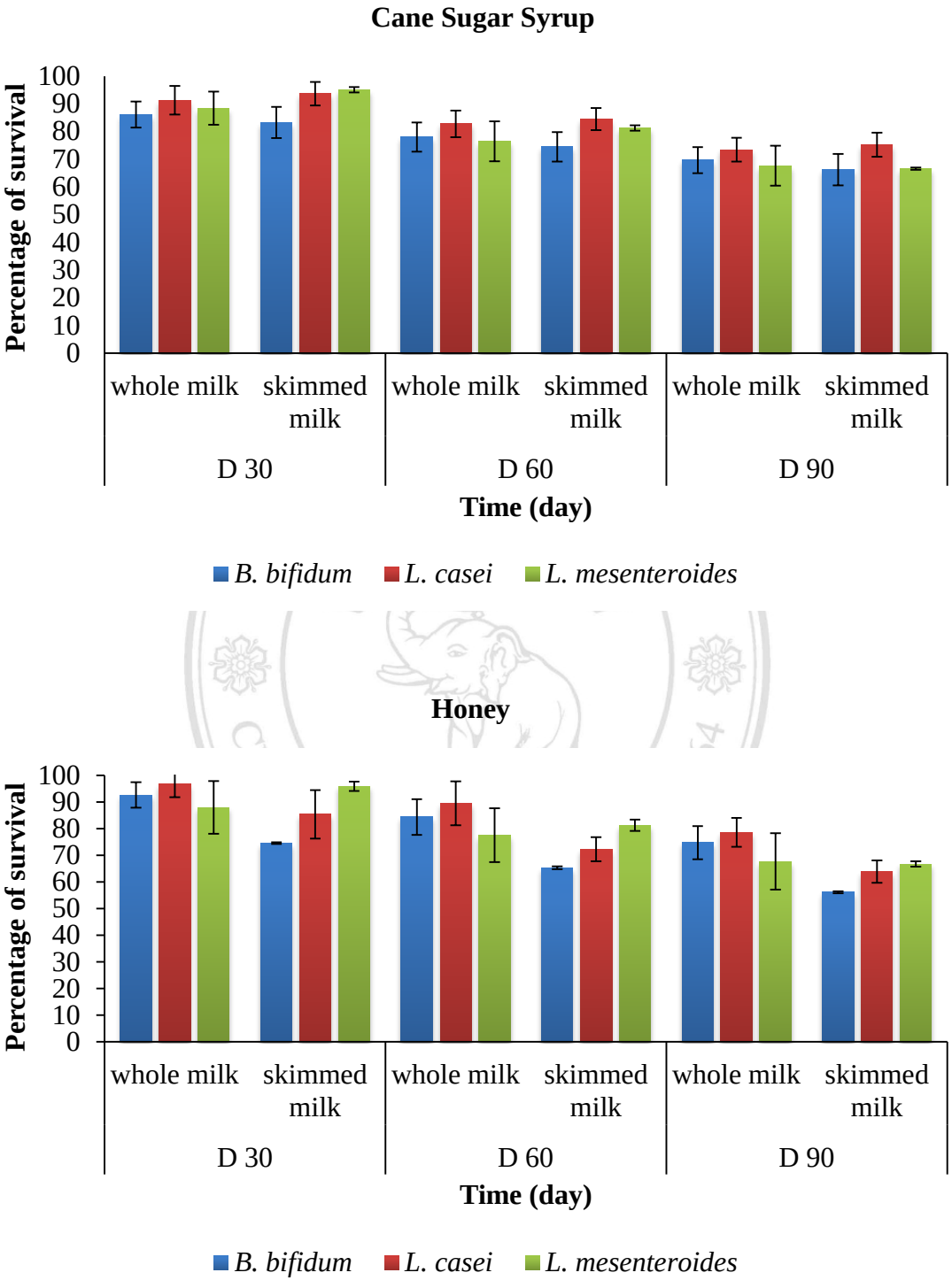

Figure 4.5 Effects of milk fat contents and types of sweeteners on survival of probiotic bacteria in yogurt ice cream after storage at -20 °C for 90 days.

As for kefir ice cream, since it was made only with whole milk, only the effects of sweeteners were to be observed. The reason for analysing total bacteria and not just probiotic bacteria in kefir ice cream were because all the bacterial strains were combined as a mixed culture from the beginning and because all strains used in the kefir starter used in this study have been known as potential probiotics. Survival of all beneficial bacteria was analysed after 30, 60 and 90 days of frozen storage and the results are shown in Figure 4.6 and Table 4.14.

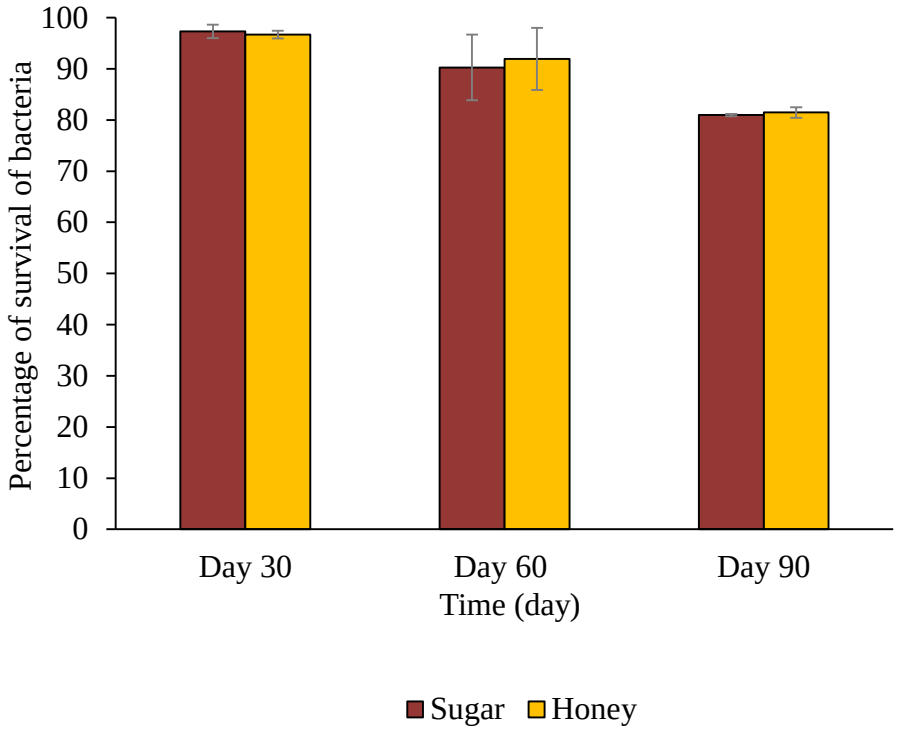

Figure 4.6 Effects of sweetener types on survival of mixed kefir culture in kefir ice cream after storage at -20 °C for 90 days.

Table 4.11 Properties of yogurt ice cream products made with different probiotic strains after storage for 30 days

Microorganisms	Milk type	Sweetener	pH	no. of probiotics in ice cream mixture (log CFU/g)	no. of probiotics in yogurt ice cream (30 days) (log CFU/g)	Survival (%)
B. bifidum	whole	sugar	4.02 ± 0.01	11.42	9.84 ± 0.53	86.14 ± 4.68 [bc]
		honey	4.00 ± 0.02	10.29	9.54 ± 0.49	92.68 ± 4.77 [c]
	skimmed	sugar	4.00 ± 0.04	10.92	9.09 ± 0.61	83.27 ± 5.61 [b]
		honey	3.97 ± 0.06	10.97	8.18 ± 0.04	74.57 ± 0.33 [a]
L. casei	whole	sugar	3.92 ± 0.02	11.68	10.66 ± 0.61	91.30 ± 5.20 [a]
		honey	3.89 ± 0.05	10.72	10.37 ± 0.52	96.70 ± 4.89 [a]
	skimmed	sugar	3.91 ± 0.01	11.05	10.35 ± 0.47	93.67 ± 4.26 [a]
		honey	3.91 ± 0.04	11.91	10.17 ± 1.08	85.39 ± 9.10 [a]
L. mesenteroides	whole	sugar	4.00 ± 0.04	10.30	9.11 ± 0.62	88.45 ± 5.97 [a]
		honey	3.94 ± 0.07	10.56	9.29 ± 1.04	87.97 ± 9.87 [a]
	skimmed	sugar	3.95 ± 0.05	7.01	6.67 ± 0.07	95.10 ± 0.93 [a]
		honey	3.91 ± 0.01	7.10	6.81 ± 0.13	95.92 ± 1.80 [a]

Data represented mean values of 3 replicates ± SD. Different letters indicate significantly difference according to Turkey ($p<0.05$)

Table 4.12 Properties of yogurt ice cream products made with different probiotic strains after storage for 60 days

Microorganisms	Milk type	Sweetener	pH	no. of probiotics in ice cream mixture (log CFU/g)	no. of probiotics in yogurt ice cream (60 days) (log CFU/g)	Survival (%)
B. bifidum	whole	sugar	3.95 ± 0.02	11.42	8.91 ± 0.60	78.02 ± 5.25 [ab]
		honey	3.93 ± 0.01	10.29	8.68 ± 0.69	84.35 ± 6.70 [b]
	skimmed	sugar	3.95 ± 0.02	10.92	8.13 ± 0.58	74.45 ± 5.31 [ab]
		honey	3.89 ± 0.02	10.97	7.17 ± 0.06	65.36 ± 0.55 [a]
L. casei	whole	sugar	3.83 ± 0.02	11.68	9.67 ± 0.56	82.79 ± 4.79 [ab]
		honey	3.81 ± 0.01	10.72	9.60 ± 0.88	89.55 ± 8.21 [b]
	skimmed	sugar	3.79 ± 0.01	11.05	9.34 ± 0.44	84.52 ± 3.98 [ab]
		honey	3.78 ± 0.03	11.91	8.61 ± 0.54	72.29 ± 4.53 [a]
L. mesenteroides	whole	sugar	3.88 ± 0.03	10.30	7.88 ± 0.74	76.50 ± 7.18 [a]
		honey	3.85 ± 0.01	10.56	8.19 ± 1.07	77.56 ± 10.13 [a]
	skimmed	sugar	3.86 ± 0.02	7.01	5.70 ± 0.07	81.31 ± 1.00 [a]
		honey	3.82 ± 0.02	7.10	5.77 ± 0.15	81.27 ± 2.11 [a]

Data represented mean values of 3 replicates ± SD. Different letters indicate significantly difference according to Turkey ($p<0.05$)

Table 4.13 Properties of yogurt ice cream products made with different probiotic strains after storage for 90 days

Microorganisms	Milk type	Sweetener	pH	no. of probiotics in ice cream mixture (log CFU/g)	no. of probiotics in yogurt ice cream (90 days) (log CFU/g)	Survival (%)
B. bifidum	whole	sugar	3.88 ± 0.02	11.42	7.96 ± 0.54	69.70 ± 4.73 [ab]
		honey	3.86 ± 0.02	10.29	7.66 ± 0.64	74.77 ± 6.22 [b]
	skimmed	sugar	3.88 ± 0.02	10.92	7.24 ± 0.62	66.30 ± 5.68 [ab]
		honey	3.84 ± 0.02	10.97	6.16 ± 0.04	56.15 ± 0.36 [a]
L. casei	whole	sugar	3.73 ± 0.01	11.68	8.58 ± 0.50	73.46 ± 4.28 [ab]
		honey	3.70 ± 0.01	10.72	8.43 ± 0.58	78.64 ± 5.41[b]
	skimmed	sugar	3.67 ± 0.02	11.05	8.32 ± 0.48	75.29 ± 4.34 [ab]
		honey	3.65 ± 0.01	11.91	7.61 ± 0.50	63.90 ± 4.20 [a]
L. mesenteroides	whole	sugar	3.78 ± 0.03	10.30	6.97 ± 0.74	67.67 ± 7.18 [a]
		honey	3.69 ± 0.02	10.56	7.15 ± 1.12	67.70 ± 10.61[a]
	skimmed	sugar	3.72 ± 0.02	7.01	4.67 ± 0.03	66.62 ± 0.43 [a]
		honey	3.67 ± 0.04	7.10	4.74 ± 0.07	66.76 ± 0.98 [a]

Data represented mean values of 3 replicates ± SD. Different letters indicate significantly difference according to Turkey ($p<0.05$)

Table 4.14 Properties of kefir ice cream after storage for 30, 60 and 90 days

Day of storage	Sweetener	pH	Total bacteria in ice cream mixture (log CFU/g)	Total bacteria in kefir ice cream (log CFU/g)	Survival (%)
30	sugar	3.93 ± 0.02	10.08	9.81 ± 0.14	97.32 ± 1.34 [a]
	honey	3.80 ± 0.03	10.12	9.78 ± 0.07	96.67 ± 0.75 [a]
60	sugar	3.81 ± 0.01	10.08	9.10 ± 0.65	90.28 ± 6.42 [a]
	honey	3.70 ± 0.01	10.12	9.31 ± 0.61	91.96 ± 6.06 [a]
90	sugar	3.71 ± 0.01	10.08	8.16 ± 0.02	80.98 ± 0.21 [a]
	honey	3.66 ± 0.01	10.12	8.24 ± 0.11	81.46 ± 1.06 [a]

* MOs = Microorganisms

Data represented mean values of 3 replicates ± SD. Different letters indicate significantly difference according to Turkey ($p<0.05$)

CHAPTER 5

Discussions

From the screening of probiotic bacteria from the fermented products in Northern Thailand, some bacterial isolates that can potentially be probiotics were found. Most of the bacterial isolates were bile tolerant, but only 38 isolates were tolerant to acid (pH 2.0) and bile salt (0.4%). On the other hand, all of the yeast isolates were able to tolerate bile (Chen *et al*., 2010). Identification of the isolates revealed that almost all of them were the members of *Lactobacillus* group, which were promising to be used in food, especially *Lactobacillus plantarum, L. brevis* and *L. paracasei*. Yeast strains that were tolerant to acid and bile salt and were potential species for food application were *Zygosaccharomyces rouxii* and *Kluyveromyces marxianus*. However, among these, only *Lactobacillus paracasei* was in the list of the probiotic microorganisms that were allowed to be used in food, according to the Guidance of Use of Probiotics in Food by the Thai Food and Drug Administration.

When considering the limitation of using new isolates of probiotic microorganisms in food, it was decided that the probiotics to be used in ice cream products in this study should be selected from the reference (known) probiotic strains available in culture collections. They were to represent *Lactobacillus* group, which colonises small intestine, and *Bifidobacterium*, which colonises large intestine. In addition, *Leuconostoc*, which may have a positive effect to the texture of the products due to its ability to produce exopolysaccharides was also selected to be incorporated in the product. Five probiotic strains including *Bifidobacterium longum* B41409, *Bifidobacterium bifidum* B4140, *Lactobacillus casei* subsp. *casei* B1922, *Lactobacillus acidophilus* TISTR2365 and *Leuconostoc mesenteroides* TISTR473 were finally selected.

From their growth characteristics, *B. bifidum*, *L. casei*, *L. acidophilus* and *L. mesenteroides* were considered suitable to be used as probiotic cultures in yogurt-based ice cream because they grew fast and could be cultured to reach a high level at a reasonable time. These are important criteria for selection of a strain to be used in a product in industrial scale or for commercial purpose. Concerning the growth rates, *B. bifidum* and *L. casei* were the most potential candidates; however, *L. mesenteroides*t was also selected due to its ability to produce exopolysaccharide, which might improve the texture of the ice cream, and its ability to grow at low temperature (De Bellis *et al*., 2010), which might allow it to survive better in yogurt ice cream.

When tested for adhesion to a modelled intestinal cell line (Caco-2), the three chosen probiotic bacterial strains and the mixed culture from kefir were found to have the the ability to adhere to cells, which is one of the key probiotic properties. However, *L. mesenteroides* had the highest adherence percentage compared to the other individual bacterium. This might be due to their ability to produce and secrete exopolysaccharides, which promotes the adhesion to the modelled intestinal cells. The mixed kefir culture was the second-best culture in terms of adhesion. Interestingly, this mixed kefir culture also include *Leuconostoc mesenteroides*, which might have contributed to the adhesion of the Caco-2 cells.

Besides the desirable properties, the selected probiotic cultures and the kefir culture were found to be suitable for use in the yogurt-based and kefir-based ice cream products. They did not create an adverse effect on flavour or texture of the ice cream products. The fat content of milk (whole and skimmed milk) did not seem to a direct effect on the survival of probiotics. The types of sweeteners (sugar and honey), however, seemed to affect the survival of some cultures. Nevertheless, the storage time was the key factors that affected survival of the probiotic bacteria; the longer the storage time is, the less numbers of live cells present in the ice cream products.

Viscosity analysis of concentrated samples showed that the concentrated yogurt made with *B. bifidum* and *L. mesenteroides* were more viscous than those made with other probiotic

strains. From the observation, the concentrated yogurt made with these cultures had thick, viscous textures, which were in accordance with the result from the viscosity test. These results may be due to the exopolysaccharides produced by organisms. The exopolysaccharides can be used as a replacement for thickening agents such as pectin and gums in yogurt (Min and Chung, 2016).

This study has developed different formulae for yogurt and kefir ice cream products, which would give the consumers alternatives, especially in terms of fat contents and the types of sugar in the products. In general terms, the fat content and the sweetener type did not seem to have a significant effect on the selected probiotic cultures under the short-term frozen storage condition, but they affected the survival of *B. bifidum* and *L. casei* in the prolonged frozen storage of 90 days.

Concerning the fat contents, there are still some disagreements among researchers whether they affect the survival of probiotics (Das *et al*., 2015). The ice cream products in this study also had other possible contribution factors to the survivals such as the cream that is an additional ingredient. However, the levels of probiotics still remained acceptably high (≥6 log CFU/g; most were in the range of 6-8 log CFU/g), indicating that the process developed in this study could be used effectively to make yogurt- and kefir-based ice cream that can be classified as probiotic products (Bandiera *et al*., 2013).

CHAPTER 6

Conclusions

In the present study, in terms of processing, *B. bifidum* B4140, and *L. casei* subsp. *casei* B1922 was suitable for use as probiotic cultures in yogurt ice cream because of their abilities to grow fast and to the sufficiently high levels to be incorporated in the yogurt ice cream products. Likewise, the mixed kefir culture used in this study was suitable for kefir-based ice cream product.

The selected probiotic bacterial strains and the mixed kefir culture also showed positive results in the adhesion assay, which support their potential health benefit. *L. mesenteroides* showed the highest percentage of adhesion to Caco2 cells, followed by the mixed kefir culture, *B. bifidum* and *L. casei*, respectively.

Fat contents or the types of milk did not seem to affect the survival of probiotics in yogurt, but the types of sweetener might have some effects on some cultures, especially *B. bifidum* and *L. casei*, as observed in our study.

The probiotic cultures decreased during the prolonged frozen storage, nevertheless, they still remained at an acceptably high level at 90 days of storage with the preparation process presented in this study. The numbers of live probiotics that were added in the ice cream mixture should be at least 10 log CFU/ml in order to remain sufficiently high (≥ 6 log CFU/g) in the yogurt ice cream to be classified as probiotic products. The same figure is recommended for kefir ice cream mixture.

One interesting notice was that when the ice cream yogurt and ice cream kefir samples were analysed for fat content, all recipes with added sugars showed higher fat contents than

those with honey added. The texture analysis showed that the yogurt made with *L. mesenteroides* and *B. bifidum* had a firmer texture.

Overall, the yogurt ice cream production process using concentrated yogurt and concentrated kefir made from either whole milk or skimmed milk and natural sweeteners reported in this study supported the survival of probiotic bacterial strains. The formulae developed in this study could be used as basic formulae for further development of probiotic ice cream products.

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APPENDIX

APPENDIX A

Culture Media Preparation

1. 0.1 % Peptone water

Peptone	1	g
Distilled water	1,000	ml
pH 7.2 ± 0.2		

Preparation

1. Peptone was dissolve in distilled water and adjust pH to the range 7.2±0.2.
2. The media was sterilized at 121 °C, pressure 15 psi, 15 minutes.

2. Potato Dextrose agar (PDA)

Potato extract	200	g
Dextrose	20	g
Agar	17	g
Distilled water	1,000	ml
pH 4.5		

Preparation

1. PDA was dissolved in distilled water and adjust pH to 4.5.
2. Then, the media was sterilized at 121 °C, pressure 15 psi, 15 minutes.
3. When a temperature decrease to 55-60 °C, the culture media was poured into a sterilized petri dish.

3. DeMan Rogosa Sharp (MRS) with Cysteine Bromophenol blue agar

Peptone	10	g
Meat extract	10	g
Yeast extract	5	g
Glucose	20	g
Tween 80	1	g
di-potassium hydrogen phosphate	2	g
Sodium acetate	5	g
Tri-ammonium citrate	2	g
$MnSO_4 \cdot 4H_2O$	0.05	g
$MgSO_4 \cdot 7H_2O$	0.2	g
Bromophenol blue	0.02	g
Cysteine	0.5	g
Agar	15	g
Distilled water	1,000	ml
pH 6.2 - 6.5		

Preparation

1. The ingredients were mixed in distilled water and adjust pH to 6.2-6.5.
2. Then, the media was sterilized at 121 °C, pressure 15 psi, 15 minutes.
3. When a temperature decrease to 55-60 °C, the culture media was poured into a sterilized petri dish.

4. Yeast extract peptone dextrose (YPD) agar

Yeast extract	10	g
Peptone	20	g
Dextrose	20	g
Agar	15	g
Distilled water	1,000	ml
pH 6.5 ± 0.2		

Preparation

1. The ingredients were mixed in distilled water and adjust pH to 6.5±0.2.
2. Then, the media was sterilized at 121 °C, pressure 15 psi, 15 minutes.
3. When a temperature decrease to 55-60 °C, the culture media was poured into a sterilized petri dish.

5. Hestrin and Schramm (HS) agar

Glucose	20	g
Peptone	5	g
Yeast extract	5	g
Na_2HPO_4	2.7	g
Citric acid	1.15	g
Agar	20	g
Distilled water	1,000	ml
pH 7.1 ± 0.2		

Preparation

1. The ingredients were mixed in distilled water and adjust pH to 7.1±0.2.
2. Then, the media was sterilized at 121 °C, pressure 15 psi, 15 minutes.
3. When a temperature decrease to 55-60 °C, the culture media was poured into a sterilized petri dish.

6. Bromocresal purple ethanol agar

Glucose	5	g
Yeast extract	10	g
Peptone	10	g
Glycerol	20	ml
Potato extract	4.5	g
Bromocresol purple	0.3	g
95% ethanol	4%	
Agar	20	g
Distilled water	1,000	ml

pH 6.8

Preparation

1. All of ingredient were mixed in distilled water (except 95% ethanol) and adjust pH to 6.8.
2. Then, the media was sterilized at 121 ℃, pressure 15 psi, 15 minutes.
3. The volume 4% of 95% ethanol was added when the temperature decreased to 55-60 ℃ and mixed.
4. The culture media was poured into a sterilized petri dish.

APPENDIX B

Chemical Preparation for Gram Stain

1. Gram stain

1.1 Crystal violet

Crystal violet	0.5	g
Distilled water	100	ml

The chemical was dissolved in distilled water and stored in a bottle.

1.2 Decolorizer

95% Ethanol	250	ml
Acetone	250	ml

The solutions were dissolved together and stored in a bottle with a brown lid.

1.3 Iodine solution

Iodine	1.0	g
Potassium	2.0	g
Distilled water	300	ml

The chemicals were dissolved together in distilled water and stored in a bottle with a brown lid.

1.4 Safranin O

Safranin O	2.5	g
Ethanol	100	ml

The chemical was mixed together and stored in a bottle with a brown lid**.**

APPENDIX C

Chemical for DNA Extraction and PCR

1. DNA extraction and amplification for yeast

1.1 DNA extraction

1.1.1 Lysis buffer

1) 2% (v/v) TritonX-100 (Amresco)
2) 1% (w/v) SDS (Vivantis, Malaysia)
3) 100 mM NaCl (Vivantis, Malaysia)
4) 10 mM Tris-HCl, pH 8.0 (Vivantis, Malaysia)
5) 1 mM EDTA, pH8.0 (Vivantis, Malaysia)

1.1.2 TE buffer

1) 10 mM Tris, pH 8.0 (Vivantis, Malaysia)
2) 1 mM EDTA, pH 8.0 (Vivantis, Malaysia)

1.1.3 Chloroform

1.2 DNA amplification reaction

1.2.1 2x MyTaq Mix (Bioline, USA)

1.2.2 Forward primer NL1 (99822421 N. Rodrassamee 228334330 NL1)

1.2.3 Reverse primer NL4 (99822422 N. Rodrassamee 228334329 NL4)

1.2.4 Distilled water

2. DNA extraction and amplification for bacteria

2.1 DNA extraction

2.1.1 Lysis buffer

0.5% (w/v) SDS	5	g
250 mM NaCl	14.61	g
200 mM Tris, pH 8.5	24.23	g
25 mM EDTA	9.31	g

Preparation

1) Each chemical was prepared separately (0.5% (w/v) SDS 5 g in DW 100 ml, 250 mM NaCl 14.61 g in DW 200 ml, 24.23 g of 200 mM Tris, pH 8.5 in 400 ml DW, and 25 mM EDTA 9.31 g in 100 ml DW).
2) All of chemical were mixed, and adjust to pH 9.0 with HCl solution, then fill up distill water to 1000 ml and sterilized at 121 °C, pressure 15 psi, 15 minutes.

2.1.2 TE buffer

10 mM Tris HCl	1.214	g
1 mM EDTA	0.3724	g

The chemicals were mixed in distill water to 1000 ml and sterilized at 121 °C, pressure 15 psi, 15 minutes.

2.1.3 25 ml Phenol : 24 ml Chloroform : 1 ml Isopropanol

2.1.4 Sodium Acetate (CH_3COONa)

$CH_3COONa \cdot 3H_2O$ 408.1 g

Adjust to pH 5.2 with glacial acetic and distill water fill up to 1000 ml

2.1.5 99.9% Ethanol absolute

2.1.6 70% Ethanol

2.2 DNA amplification reaction

2.2.1 2x MyTaq Mix (Bioline, USA)

2.2.2 Forward primer 27F (5'-AGAGTTTGATCMTGGCTCAG-3')

2.2.3 Reverse primer 1492R (5'-GGTTACCTTGTTACGACTT-3')

2.2.4 Distilled water

3. Chemicals for agarose gel electrophoresis

3.1 1% agarose gel (Merck, Germany)

3.2 1X TAE buffer

3.3 Save view (NEOgreen, Korea)

3.4 5X or 6X Loading dye (Vivantis, Malaysia)

3.5 VC 1 kb DNA Ladder (Vivantis, Malaysia)

APPENDIX D

Chemical Preparation for Cell Culture

1. Giemsa stain

1.1 0.38% Giemsa was fused with 50 ml of 99.9% methanol, and 50 ml of glycerol.

1.2 The mixed solution was shacked at room temperature an overnight.

1.3 Then, the mixed solution was filtered to new tube by 0.22 µm and wrap with aluminum foil.

2. Gentamycin

2.1 2 mg/ml of gentamycin was dissolved in sterile DI water.

2.1 The solution was filtered by 0.22 µm and keep at 4 °C.

3. MTT

2 mg/ml of MTT was dissolved in sterile 1X PBS and keep at 4 °C.

4. 1X Phosphate Buffer Saline (PBS)

NaCl	8	g
KCl	200	mg
Na_2HPO_4	1.44	g
KH_2PO_4	245	mg
pH 7.4		

Preparation

1) All of chemical were added in 800 ml of distilled water.

2) Adjust solution to desired pH 7.4 and add distilled water until volume is 1000 ml.

5. 10% Fetal bovine serum (FBS)

6. **DMEM**

7. **DMSO solution**

8. **0.25% Trypsin EDTA**

9. **Penicillin/Streptomycin solution (100X)**

10. **Antibiotic**

APPENDIX E

Adhesion to Epithelia Cells Test

1. Preparation of Caco-2 cells culture

Subculture of Caco-2 cells

1.1 Caco-2 cells were subculture aged 2 days.

1.2 The media was removed and washed with 5 ml of 1X PBS about 2 times.

1.3 Add 0.05% trypsin 500 µl, and leave for 3-5 min

1.4 Add DMEM + 10% fetal bovine serum (FBS) media 2.5 ml and remove the media 2 ml

1.5 Add DMEM + 10% FBS media for 5 ml and Penicillin-Streptomycin 1 ml, incubate at (37 °C, 5% CO_2, 95% humidity) for 24 h

1.6 The media removed from 1.4 was centrifuged 1200 rpm, 5 min at 25 °C.

1.7 The pellet cells were added with 1 ml of DMEM + 10% FBS media.

1.8 The cells in 1.7 were diluted 10 µl in 90 µl of DMEM + 10% FBS media and counted in a Neubauer hematocytometer chamber.

1.9 The density of cell around 10^5 cells/ml.

2. Preparation of bacteria

Probiotic strains were enriched in 10 ml of MRS broth

↓

Incubate at 37 °C for 48 h in anaerobic conditions

↓

Centrifuge at 7,000 rpm for 10 min

↓

Pellet was washed with 1X PBS 2 times

↓

Adjust the inoculum to 0.1-0.2 with 1X PBS at OD600 nm

↓

Centrifuge at 7,000 rpm for 10 min

↓

Add the volume of DMEM + 10% FBS media equal 1X PBS that was using to adjusted

↓

The number of initial inoculum for adhesion as 10^8 CFU/ml

↓

The number of initial inoculum was diluted to 10^6, 10^7, and 10^8 CFU/ml in DMEM + 10% FBS media

↓

Each diluted the initial inoculum was inoculated in 6-well plate

3. The adhesion process

DMEM + 10% FBS media 3 ml, and 2 ml of Caco-2 cells (from 1.9) were added in the 6-well plate that has coverslips, and 6-well plate no coverslip.

↓

Incubate at (37 °C, 5% CO_2, 95% humidity) for 24 h

↓

The media was removed, add DMEM + 10% FBS media 1 ml, and add the cells each dilution (10^6, 10^7, and 10^8 CFU/ml)

↓

Incubate at (37 °C, 5% CO_2, 95% humidity) for 2 h

↓

3.1 Coverslips

3.1.1 Coverslips were fixed with 99.9% methanol 3 ml for 5 min and methanol removed.

3.1.2 Giemsa 3 ml was using as gram stain for 15 min.

3.1.3 Giemsa was removed, and coverslips were absorbed with tissue paper.

3.1.4 The coverslips were dried for overnight, and taken under microscope.

3.2 No Coverslips

3.2.1 300 µl of 0.05% trypsin and DMEM + 10% FBS media 700 µl were added to 6-well plate.

3.2.2 MRS-Cys-BPB agar was used to detect the adhesion to cells of probiotic strains by the drop plate technique.

3.2.3 The colonies of probiotics that appear on MRS-Cys-BPB agar were calculated for percent to ability adhesion.

$$\% \text{ Adhesion} = \frac{\text{The number of bacteria present} \times 100}{\text{Concentration of initial inoculum bacteria}}$$

APPENDIX F

Acid and Bile salt Tolerance Test

1. Preparation of microorganisms

1.1 The colonies that appear on MRS-Cys-BPB agar were transferred to MRS broth and incubated at 37 °C for 48 h in anaerobic conditions.

1.2 The cultures were adjusted the OD600 nm about 0.1-0.2 with MRS broth.

2. Acid tolerance test

2.1 MRS broth was adjusted with HCl to pH 2.0.

2.2 The broth media was sterilized at 121 °C, pressure 15 psi, 15 minutes.

2.3 Each culture from 1.2 was transferred to the MRS broth pH 2.0 and incubated at 37 °C for 3 h in anaerobic conditions.

2.4 Then, the drop plate technique was used to select the survival of microorganisms on MRS-Cys-BPB agar.

3. Bile tolerance test

3.1 MRS broth was added 0.4% ox gall and mixed.

3.2 The mixed media was sterilized at 121 °C, pressure 15 psi, 15 minutes.

3.3 Each culture from 1.2 was transferred to the MRS broth mixed with 0.4% ox gall and incubated at 37 °C for 3 h in anaerobic conditions.

3.4 Then, the drop plate technique was used to select the survival of microorganisms on MRS-Cys-BPB agar.

4. Acid + Bile tolerance test

4.1 The cultures that survived in the acid tolerance test and bile tolerance test were transferred to MRS broth that added 0.4% ox gall and adjusted the pH 2.0 with HCl.

4.2 The cultures were incubated at 37 °C for 3 h in anaerobic conditions.

4.3 Then, the drop plate technique was used to select the survival of microorganisms on MRS-Cys-BPB agar.

4.4 The cultures that survived from the test were identified by molecular technique.

APPENDIX G

Microbiological Calculation

Spread plate technique

$$\text{Microbial quantity (CFU)} = \frac{\text{the number of colonies} \times 10}{\text{Dilution factor}}$$

Drop plate technique

$$\text{Microbial quantity (CFU)} = \frac{\text{the number of colonies} \times 100}{\text{Dilution factor}}$$

APPENDIX H

Viscosity Analysis

Methods

1) Open the computer and program
2) Calibrate force and setting the test value
3) Place the sample in glassware on the platform
4) Run a test
5) Data analysis from the graph

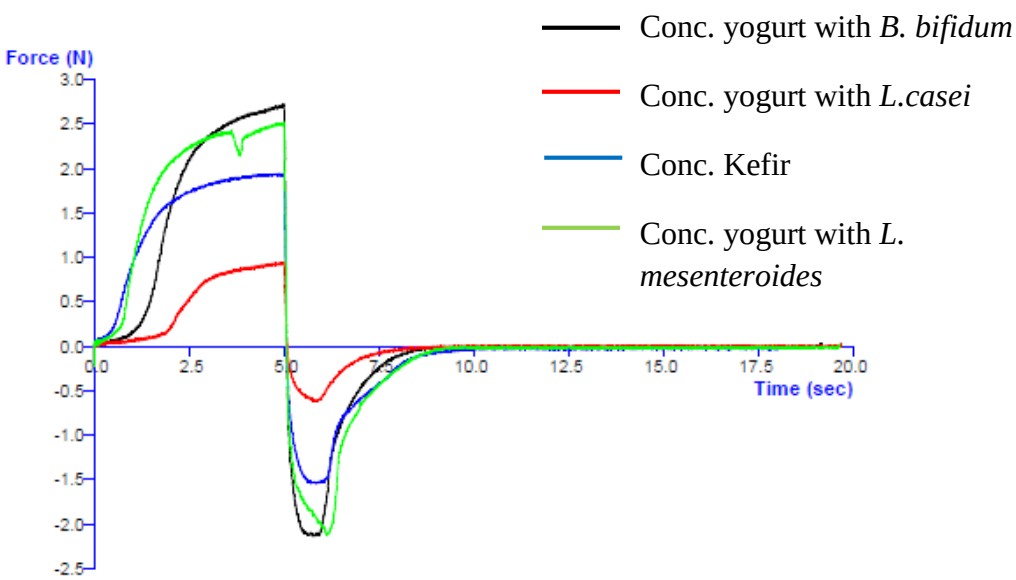

Figure H Viscosity of concentrated yogurt of probiotic 3 strains and kefir

APPENDIX I

Analysis of Fat Content by Soxhlet Extraction Method

Methods

1) Cups and samples 1 g (on whatman paper) were taken to hot air oven at 60 °C for 16 h.
2) Put the cup in the desiccator until the temperature is equal to room temperature.
3) Cups were weighted and taken to hot air oven at 60 °C for 30 min.
4) Put the cup in the desiccator until the temperature is equal to room temperature.
5) Cups were weighted 3 repeats and calculated the average.
6) The samples in 1) were put in thimbles and connect to Soxhlet.
7) Petroleum ether 50 ml were added to extraction cups.
8) The extraction cups were connected with thimbles on Soxhlet.

9) Setting the program for extraction is 90 °C, over temperature of petroleum ether is 145 °C, boiling 30 min, rinsing 1 h, recovery 15 min, and drying 3 min.

10) Cups were taken to hot air oven at 60 °C for 1 h.

11) Put the cup in the desiccator until the temperature is equal to room temperature.

12) Cups were weighted and taken to hot air oven at 60 °C for 30 min.

13) Put the cup in the desiccator until the temperature is equal to room temperature.

14) Cups were weighted 3 repeats and calculated the average.

15) Calculate the analysis results as follow:

$$\% \text{ fat} = \frac{(\text{cup weight after extraction} - \text{cup weight before extraction}) \times 100}{\text{Sample weight}}$$

Curriculum Vitae

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