# คุณลักษณะของโพรไบโอติกแบคทีเรียกรดแลกติกที่สร้างเอนไซม์ไบล์ซอลท์ไฮโดรเลสในการพัฒนาผลิตภัณฑ์นมหมัก

# Characterization of probiotic lactic acid bacteria producing bile-salt hydrolase for development of fermented milk product

PORNCHANOK PAONGPHAN

GRADUATE SCHOOL Srinakharinwirot University

2018

# คุณลักษณะของโพรไบโอติกแบคทีเรียกรดแลกติกที่สร้างเอนไซม์ไบล์ซอลท์ไฮโดรเลส ในการพัฒนาผลิตภัณฑ์นมหมัก

พรชนก ป้องพาล

ปริญญานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตร
วิทยาศาสตรมหาบัณฑิต สาขาวิชาชีวภาพการแพทย์
คณะแพทยศาสตร์ มหาวิทยาลัยศรีนครินทรวิโรฒ
ปีการศึกษา 2561

# Characterization of probiotic lactic acid bacteria producing bile-salt hydrolase for development of fermented milk product

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A Thesis Submitted in partial Fulfillment of Requirements

for MASTER OF SCIENCE (Biomedical Sciences)

Faculty of Medicine Srinakharinwirot University

2018

THE THESIS TITLED

CHARACTERIZATION OF PROBIOTIC LACTIC ACID BACTERIA PRODUCING BILE-SALT HYDROLASE FOR DEVELOPMENT OF FERMENTED MILK PRODUCT

BY

PORNCHANOK PAONGPHAN

HAS BEEN APPROVED BY THE GRADUATE SCHOOL IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE MASTER OF SCIENCE IN BIOMEDICAL SCIENCES AT SRINAKHARINWIROT UNIVERSITY

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| Degree | MASTER OF SCIENCE |
| Academic Year | 2018 |
| Thesis Advisor | Assistant Professor Malai Taweechotipatr , Ph.D. |

Current studies have suggested that probiotic lactic bacteria confers health benefits to the human. One of the benefits of probiotic lactic acid bacteria they can reduce cholesterol by synthesizing bile salt hydrolase. In this study, 55 Isolates of lactic acid bacteria from fermented food were selected to investigate for their potential bile salt hydrolase activity and evaluated for their application in milk product.

In the present study, three isolates including MN, MN2 and SM exhibited the capability to produce the strongest bile salt hydrolase activity. The species identification of these lactic acid bacteria used 16S rRNA gene sequence analysis. It showed that isolate MN was *Lactobacillus paraplantarum*, isolate MN was *Lactobacillus plantarum* and isolate SM was *Lactobacillus gasseri*. After that, the result showed three-strain exhibited good resistance to pH 3.0 and bile concentration at 0.3% and 0.8%. At pH2 all isolates did not survive. All 3 isolates show moderate hydrophobicity. Antibiotic sensitivity tests showed that three isolate showed resistance to nalidixic acid and streptomycin. In addition, the antimicrobial activity against pathogens isolate MN showed low inhibition against *Shigella dysenteria* and isolate SM showed low inhibition against *Vibrio parahaemolyticus* and *Shigella dysenteria*. All the three isolates were incapable of exhibiting hemolysis activity. In view of their application in milk product it was found that isolates MN, MN2, SM and Mixed culture could maintain bile salt hydrolase activity in fermented milk products. Thereby, with the properties of good probiotics these strains could be potentially used in health products especially where cholesterol reduction by enzyme bile salt hydrolase in food is the main target.

Keyword : Probiotic lactic acid bacteria Bile salt hydrolase Fermented milk products

# ACKNOWLEDGEMENTS

The success and final outcome of this project required a lot of guidance and assistance from many people and I am extremely privileged to have got this all along the completion of my project. All that I have done is only due to such supervision and assistance and I would not forget to thank them.

I respect and thank my advisor, Assst. Prof. Malai Taweechotipatr Ph.D. and co-advisor, Assst. Prof Ulisa Pachekrepapol Ph.D. for providing me an opportunity to do the project on the topic " Characterization of probiotic lactic acid bacteria producing bile-salt hydrolase for development of fermented milk product" and giving us all support and guidance which made me complete the project duly. I am extremely thankful to Assst. Prof. Malai Taweechotipatr and co-advisor, Assst. Prof Ulisa Pachrepapol for providing such a nice support and guidance, although she had busy schedule managing the corporate affairs.

I also would like to thank the Department of Microbiology, Faculty of Medicine, Srinakharinwirot University for my research. Researchers and staffs Faculty of Pharmaceutical Science, Srinakharinwirot University and Faculty of Pharmaceutical Science, Chulalongkorn University for their kind suggestions, helpfulness and encouragement.

Finally, i would also like to thank my parents and friends who helped me a lot in finalizing this project within the limited time frame.

PORNCHANOK PAONGPHAN

# TABLE OF CONTENTS

Page

# LIST OF TABLES

Page

# LIST OF FIGURES

Page

# CHAPTER 1
# INTRODUCTION

Probiotics are known as good microorganisms and have health benefits. The World Health Organization (WHO) defines probiotics as "live microorganisms which when administered in adequate amounts confer a health benefit on the host". Most probiotics are lactic acid bacteria (LAB) that have been studied for a long time, and most of them are non-pathogenic.[1] The common sources of probiotics are fermented foods, yoghurt, cultured buttermilk, cheese and are also found in intestinal microbes. The greater part of probiotic microorganisms belongs to the genus *Lactobacillus* and *Bifidobacterium* both are anaerobic bacteria, gram-positive, lactic acid–producing bacteria that constitute important intestinal microflora. Other microbes may also be used as probiotics, such as *Lactococcus, Leuconostoc, Enterococcus* and yeast *Saccharomyces boulardii*. [2] Several species of lactobacilli have generally regarded as safe status (GRAS) and some can interact with intestinal epithelial cells.

Probiotics are often administered for treatment of intestinal disorders, such as diarrhea and its alleviation. [3] The main route of probiotic administration is mouth carriage to enter the gastrointestinal system, the most key target for probiotics action. The benefits of probiotics include modulation of immune function (immunomodulation), inhibition of pathogens (anti-pathogenic), reduction of the symptoms of allergies (anti-allergy), reduction of inflammation (inflammatory bowel disease; IBD), anti-cancer and reduction of lipid level in serum (hyperlipidemia). Good probiotics must have beneficial effect on the host, contain a large number of viable cells at the time of consumption, remain viable throughout the shelf-life of the product and stabilize the intestinal microflora.

LAB are gram-positive bacteria, which produces lactic acid, play an important role in food production and health maintenance. The general characteristics of LAB are lactic acid production, gram-positive, catalase negative, non-motile, non-respiring and non-spore forming cocci or rods.[4] The general microorganisms recognized as LAB

group are *Aerococcus*, *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus* and *Weissella*, some other microbes have been proposed as and are used as probiotic strains include *L. acidophilus*, *L. casei*, *L. johnsonii L. fermentum*, *L. rhamnosus*, *L. plantarum*, *L. reuteri*, *L. salivarius*, *L. paracasei*, *L. delbrueckii* subsp. bulgaricus, *Saccharomyces boulardii*, *Streptococcus thermophilus*, *Bifidobacterium lactis*, *B. longum*, and *B. breve*.[5]

The human body have tremendous number of living microorganisms. The number of microbial cells inhabiting body is estimated to surpass the human body cells by 10-fold and estimated at 350 trillion microbial cells. [6] All microorganisms in the body are called microbiota. These microorganisms may be bacteria, viruses, fungi or other types of microorganisms. Various species of microorganisms are located in different parts of the body such as oral cavity, throat, esophagus, stomach, colon, urogenital tract, respiratory tract, and skin. [7] Microbiota are an important key for maintaining homeostasis where it confers many usefulness for the host such as development of the immune system, pathogen elimination, vitamin production, and absorption of nutrients. [8]

The intestine microbiota constitutes the most plentiful microbial domain within the human body. The intestine is the area where microbes live most, with up to $10^{14}$ cells. [6] Gut microbiota plays a major role of human health. It helps in digestion and absorption of food, metabolic system and the excretory system. [9, 10] Some types of gut microbiota help create a substance necessary to thrive in the body, such as vitamins B and K, stimulate and strengthen the body's immune system and protects the body from invasion by the outside pathogenic microbes. The gut is largest immune organ. The gut immune system is key to maintaining a healthy gut as well as overall health. Epithelial cells in the intestine contribute to maintain a symbiotic relationship between the gut microbiota and host by establish mucosal barriers, secreting immunological mediators, and delivering bacterial antigens. Various studies have shown a wide variety of good bacteria (probiotic) in the gut that can enhance immune system function, help combat

obesity, and provide numerous other benefits. However, when one of these bacterial colonies is out of balance, it can lead to dysbiosis.

There are many studies show that dysbiosis of the gut microbiota is associated with the pathogenesis of such chronic gastrointestinal diseases such as inflammatory bowel disease (IBS) and irritable Bowel Syndrome (IBD), and systemic metabolic diseases, such as cardiovascular disease, type 2 diabetes and obesity.[11, 12] Consumption of high-sugar, high-fat foods and chronic stress is a major cause of imbalance gut microbiota. Probiotic and Prebiotic are diet processes or strategies used as food to promote host health by improving the composition of the intestine microbiota. Prebiotic is a nutrient that stimulates the proliferation and or function of “beneficial" bacteria in the colon including probiotic microorganisms. Thus, conferring benefits upon host health. The consumption of probiotics, prebiotics and nutrients, beneficial molecules or microbes are designed to be beneficial to the body by increasing the number of beneficial microorganisms or products they have with the intestines. The balance of gut microbiota can be achieved by eating nutritious foods and eating a prebiotic or probiotic. Therapeutically, probiotics have been used to improve gut microbiota for centuries.[13, 14]

Cholesterol is a type of fat in wax and water insoluble form and sorts to be substance sterol that important for the body. Cholesterol is a part of the cell wall structure that regulates the flow of fluid between cells and it is complement of the myelin sheath. In addition, cholesterol is a precursor to synthesis the bile acids, vitamin D and important hormones in the body, including hormones of the adrenal glands, cortisol, corticosteroids, estrogen and testosterone. It is also a precursor of important substances in bile (bile salt), which helps digestion and absorption of fat.[15] Seventy percent of cholesterol in the body are made by liver and anot her 30% comes from the diet. Cholesterol is mostly found in egg yolks, animal innards and seafood.[16]

Hypercholesterolaemia is a condition, of which the body has high level of lipids in blood with more than 200 mg/dl and the level of triglyceride is higher than 150 mg/dl.[17] This condition increases fatty deposits in arteries and the risk of blockages. The main

cause of severe hyperlipidemia due to lifestyle and unhealthy eating habits such as those high in salt and fat, especially saturated fat, and low in complex carbohydrates. [18] Consumption of high cholesterol diet causes atherosclerosis, artery wall thickness or more narrow arteries which results in blood to the heart is insufficient. It is a common cause of heart attack and stroke. [19] Therefore, various strategies have been employed to relieve hypercholesterolemia such as exercise, various dietary approaches including the use of probiotics in the development of functional foods.

Bile is a fluid produced by the liver from cholesterol as a precursor in the synthesis. In humans, bile production is about 400-700 cc. [20] The gall bladder stores and concentrates bile during the fasting state. Bile contains bile acids, which important for digestion and absorption of fats and fat-soluble vitamins in the small intestine. Bile acids are derivatives of cholesterol synthesized in the hepatocyte. Cholesterol, ingested as part of the diet or derived from hepatic synthesis is converted into the bile acids cholic and chenodeoxycholic acids, which are then conjugated to an amino acid (glycine or taurine) to submit the conjugated form that is actively secreted into bile canaliculi. When eating food, bile is released from the gall bladder to duodenum thus entering the enterohepatic circulation. Reabsorbed bile acids enter the portal bloodstream and are taken up by hepatocytes, reconjugated, and re-secreted into bile. Approximately 5% of the total bile acid are lost in the feces.

In intestinal, conjugated bile salts is extensively modified by the indigenous intestinal bacteria. One important transformation is deconjugation.[21] Deconjugation is catalyzed by bacterial enzymes known as bile salt hydrolases (BSH), which transform conjugated bile salts into deconjugated bile salts by hydrolysis of the amide bond in conjugated bile salts, resulting in the release of free amino acids. The insoluble substance in conjugated bile salt is precipitated and excreted from the body with feces causing de novo bile salt synthesis from cholesterol. This mechanism is used to maintain bile acid homeostasis, which will result decrease cholesterol in blood. [22, 23]

BSH is an enzyme produced by diverse gut bacterial species in gastrointestinal tract. BSH catalyzes the glycine- or taurine- linked bile salt deconjugation reaction.[24,

[25]Commensal bacteria frequently produce BSH enzymes whose roles in their survival and colonization. [26] The main beneficial effect of bile salt deconjugation includes lowering of serum cholesterol levels, as the less amphiphilic molecules (unconjugated bile acids), which compromises fat digestibly and recycling of deconjugated bile acids forces an increase in the de novo synthesis of bile acids utilizing cholesterol.[27]

Microorganism that have been found to produce BSH activity include: *Bifidobacterium*, *Enterococcus*, *Lactobacillus*, *Enterococcus*, *Clostridium*, and *Bacteroides* spp. *Lactobacilli* and *Bifidobacteria* are most commonly used as probiotic, while *Bacteroides*, *Clostridium*, and *Enterococcus* spp. are also commensal bacteria inhabitants of the gastrointestinal tract. [28] Many LAB probiotics are found to produce BSH which beneficial effect to serum cholesterol lowering thus, BSH activity is also considered criterion for the selection of probiotics. [29]

The concept of functional foods was first used in japan in the 1980s [30] and it is connected with the notion that foods not only provide basic nutrition which is necessary for living but can also prevent diseases, good health and longevity. [31] Probiotic food has a large commercial interest and its market share has increased.[32] The presence of probiotics in health food products has been claimed for health benefits. The combination of probiotics in food products enhances their market value. Consumers commonly recognize them as a food with proven health benefits or functional food. Probiotic products are dietary supplements that contain beneficial microorganisms and affect the health of consumers. Microorganism frequently used in probiotic products are similar to those found in the intestinal tract, including *Lactobacillus*, *Bifidobacterium* and *Streptococcus*. [33] The minimum probiotic requirement for a given product is $10^6$ CFU / g of foods. [34]

Consumption of probiotic products improves intestinal microflora. Most of the probiotic-products are in the form of dairy foods (Yogurt, cottage cheese, and fermented milk). Dairy products were the first commercialized products in group of probiotics food products that are still consumed in larger quantities than other probiotic foods.[35] The usefulness on human health of probiotic dairy products includes: reducing lactose

intolerance, cholesterol lowering, prevention of diarrhea and constipation, increase in the effectiveness against *Helicobacter pylori* infection, enhancement of mineral absorption. Presently, probiotics are added to commercialized food products, such fermented vegetable juices, cereals, chocolate, ice cream, and cookies.

The aim of this study is to characterize lactic acid bacteria isolated from Thai fermented foods. The selected LAB were screened for potential with bile salt hydrolase activity, characterized by 16S rRNA gene sequencing and evaluated for the probiotic properties. In addition, the technological potential of lactic acid bacteria isolated from Thai fermented foods in view of their application in fermented milk products were determined.

## Hypothesis

Certain strains of probiotic lactic acid bacteria are able to producing bile salt hydrolase and can be applied in fermented milk products.

## Objective

1. To isolate and select the probiotic lactic acid bacteria isolated from Thai fermented food.
2. To screen probiotic lactic acid bacteria that have bile salt hydrolase activity.
3. To characterize bacterial species by 16S rRNA gene sequencing.
4. To evaluate the probiotic properties.
5. To determine the technological potential of lactic acid bacteria isolated from Thai fermented foods in view of their application in milk fermented product

# CHAPTER 2
# LITERATURE REVIEW

## 1. Definition of probiotics

Probiotics are non-pathogenic microorganisms that are beneficial to the body. Several studies have reported that probiotic bacteria play important roles in the modulation of gastrointestinal and immunological functions. The term "probiotic" was first used by Lilley and Stillwell in 1965.[36] The concept of beneficial microbes prior to probiotic originated with the concept of the Russian scientist Elie Metchnikoff, who won the Nobel Prize in the early 20th century, in 1908. He noted that the Bulgarian people had long healthy life resulted from their consumption of fermented milk products. He believed that consumption of the fermented milk indubitably influenced the gut microflora, decreasing the toxic microbial activity of the pathogenic bacterial population.[37] However, the definition of probiotic most commonly accepted is the one developed by the WHO/FAO working group in 2002: "Live microorganisms which when administered in adequate amounts confer a health benefit on the host."[38]

### 1.1 Common probiotics

The most essential probiotic properties must be able to survive and colonize in the human gastrointestinal tract, and thus must be able to tolerate pH and concentration of bile acids. In addition, should be recognized by the immune system. It should not be toxic, allergic and non-pathogenic. The probiotics strains for human use should have 'generally regarded as safe (GRAS)' status, which focus on strain identification, functional properties, safety assessment, and beneficial human health effects.[39, 40] A basic schematic diagram on the properties of an ideal probiotic can be found in Fig. 1

### 1.2 Sources of probiotics

The common sources of probiotics are fermented foods such as Japanese miso, tempeh, yogurt, cultured buttermilk, and cheese. However, probiotic strains are also found in non- fermented substrates which includes legumes, cabbage, maize, pearl

millet, cereals, and so forth.[41] The other sources of probiotics include breast milk, the human gastrointestinal tract and the guts of several animal.[42]

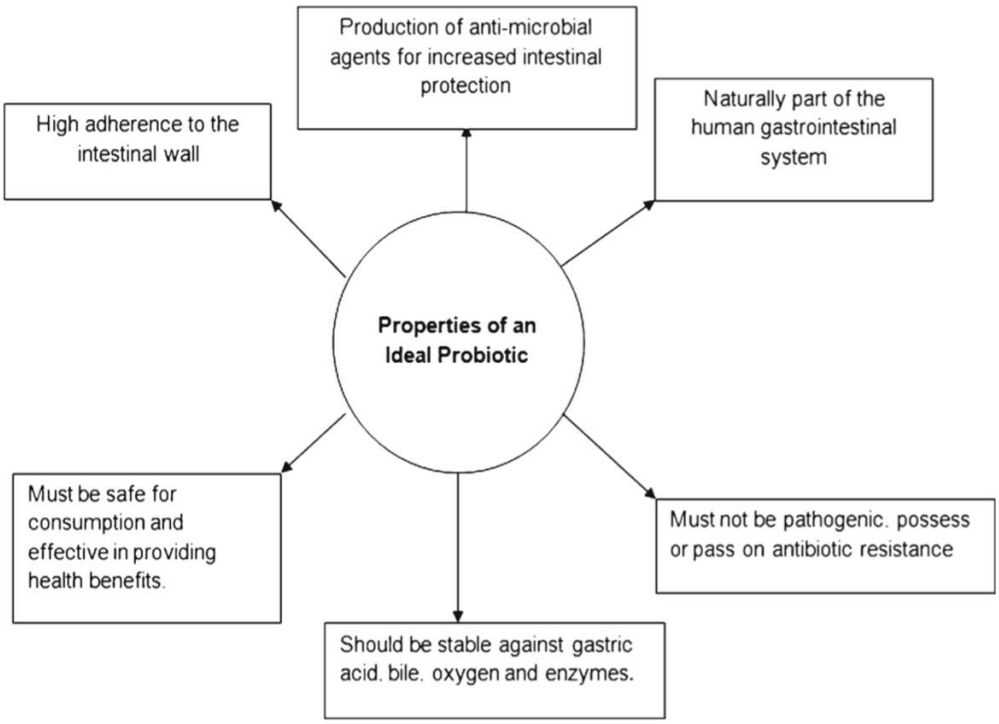



Figure 1 Basic schematic detailing the properties of an ideal probiotic bacteria [2]

### 1.3 Strain of probiotics

Most probiotic strains are lactic acid bacteria. *Lactobacillus* and Bifidobacterium both are anaerobic bacteria, gram-positive, lactic acid–producing bacteria that constitute important of intestinal microflora. Other microbes may also be used as probiotics, such as *Lactococcus, Leuconostoc, Enterococcus* and yeast *Saccharomyces boulardii*.[43] Furthermore, the selection of probiotic use in humans, should consider the ability to provide health benefits; promote or maintain the state of well-being; and safety assessment, long-term effects and also possible chronic effects.

#### 1.3.1 *Lactobacillus*

*Lactobacillus* is the main genus within the group of lactic acid bacteria. They are gram positive rod, facultative anaerobic or microaerophilic, non-spore-forming

bacteria of the family *Lactobacillaceae*. *Lactobacillus* are commensal inhabitants of gastrointestinal tracts, female urogenital tract and human mouth. *Lactobacillus* are generally used in the manufacture of fermented dairy and interest in their applications is reinforced by documented probiotic and functional properties. Some of these microorganisms have the special aptitude to produce substance that possess functional as well as therapeutic properties.[44] It was shown that *L. acidophilus* LA1 having antimicrobial effect against Helicobacter pylori, both in vitro and in humans.[45, 46]

1.3.2 *Bifidobacterium*

The genus *Bifidobacterium* are branched anaerobic bacteria, non-motile, non-spore forming, and non-gas producing. They are lactic acid bacteria that naturally inhabit our intestinal and urogenital tracts. Given their sensitivity to oxygen *Bifidobacterium*, in the gastrointestinal tract, are principally located in the colon.[47]

Table 1 Microorganisms considered as probiotics [48, 49]

| *Lactobacillus* species | *Bifidobacterium* species | Other lactic acid bacteria | Non-lactic acid bacteria |
|---|---|---|---|
| *L. acidophilus* | *B. bifidum* | *Enterococcus faecalis* | *Escherichia coli* Nissle |
| *L. casei* | *B. breve* | *Enterococcus faecium* | *Propionibacterium freudenreichii* |
| *L. crispatus* | *B. lactis* | *Lactococcus lactis* | |
| *L. gasseri* | *B. longum* | *Leuconostoc mesenteroides* | *Saccharomyces cerevisiae* |
| *L. fermentum* | *B. infantis* | | *Saccharomyces boulardii* |
| *L. johnsonii* | *B. adolescentis* | *Pediococcus acidilactici* | |
| *L. paracasei* | *B. animalis* | *Streptococcus thermophilus* | |
| *L. plantarum* | | | |

## 2. Probiotic properties

The helpful effects of probiotics on human health and nutrition are extremely recognized by health professionals. Various studies have reported the good effects of specific strains of probiotics on the reduction of risks and management of a range of diseases and conditions.[46]

### 2.1 Anti-pathogenic properties

Antimicrobial activity is an important mechanism for probiotic to competitively exclude or inhibit invading microorganisms. Some species of probiotic bacteria are capable of producing antimicrobial compounds, such as short-chain fatty acids, bacteriocins and hydrogen peroxide. Probiotics are major lactic acid producers. Lactic acid reduces pH in the area and inhibits the growth of bacteria sensitive to acidic conditions.[50, 51]

### 2.2 Adhesion properties

Bacterial adherence to the host gastrointestinal tract has long been considering important selection criteria for probiotic microorganisms. Probiotic adhesion to intestinal mucus and epithelial cells is one of the most important characteristics for host colonization. Adhesion to the intestinal mucosa leads to direct interactions that may affect the competitive exclusions of pathogens and host immune response modulation.[52]

### 2.3 Tolerance to gastric acid and bile tolerance

The resistance to acid and bile in the human gastrointestinal tract constitutes key selection criteria for probiotic bacteria. Probiotic bacteria must be able to tolerate in acidic gastric environment and high concentrations of both conjugated and deconjugated bile acids, to survive and colonize in the gut. [49] Many *in vitro* and *in vivo* studies show that probiotics organisms can survive the gastric transit, where the cells were exposed to acidic pH values < 2.0, though the exposure time was relatively short (1 to 2 h).[53]

### 2.4 Competitive exclusion of pathogenic microorganism properties

Probiotics are able to exclude or eliminate the growth of pathogens by creation of a hostile microenvironment like the decreasing of the pH of the intestine lower than what is essential for survival of pathogenic microorganisms such as *E. coli*, and *Salmonella* by producing organic acids like lactic acid.[54]

## 3. Health benefits of probiotic bacteria

Nowadays, it is widely accepted that the indigenous microbial communities are host specific, location specific, most complex in composition and has beneficial characteristic to the host. Some of the major health features of probiotics are discussed in the following sections. The major health benefits of probiotics and their proposed mechanisms are shown in Table 2.

Table 2 Health benefits of probiotic bacteria to the host, and speculated mechanisms involved [55]

| Health benefits | Proposed mechanisms involved |
|---|---|
| Identification of digestion Small bowel bacterial overgrowth | Bacterial lactase acts on lactose in the small intestine Lactobacilli influence the activity of overgrowth flora, decreasing toxic metabolite production<br>Antibacterial characteristics |
| Antihypertensive effect | Antihypertensive effect<br>Bacterial peptidase action on milk protein results in antihypertensive tripeptides<br>Cell wall components act as ACE inhibitors |

Table 2 (continued)

| Health benefits | Proposed mechanisms involved |
|---|---|
| Infection caused by *Helicobacter pylori* | Competitive colonization Inhibition of growth and adhesion to mucosal cells, decrease in gastric *Helicobacter pylori* concentration |
| Immune system modulation | Strengthening of nonspecific and antigen-specific defense against infection and tumors<br>Adjuvant effect in antigen-specific immune responses<br>Regulating/influencing Th1/Th2 cells, production of anti-inflammatory<br>Cytokines Decreased release of toxic N-metabolites |
| Urogenital Infections | Adhesion to urinary and vaginal tract cells competitive Exclusion Inhibitor production ( bacterocin, $H_2O_2$, biosurfactants) |

**3.1 Acute infectious diarrhea**

Acute infectious diarrhea is still a major health problem worldwide and a cause of death for children in underdeveloped countries. The cause of acute infectious diarrhea is caused either due to bacteria or viruses. The effects of probiotics in reducing the risk and duration of diarrhea include an elevation of the body's immune system and producing antibodies against causative microbes, such as rotavirus and *E. coli.*[56]

### 3.2 *Helicobacter pylori* infection

*Helicobacter pylori* is a gram-negative, flagellated organism that causes infection in the stomach. *H. pylori* can survive in gastric mucosa and it is linked to gastric carcinoma, gastric ulcers, and many other gastric problems. [(22)(22)] Several studies have evaluated the effects of probiotics on *H. pylori*. A recent study showed that *L. johnsonii* La1, *L. salivarius* and *L. acidophilus*, inhibit the attachment of *H. pylori* to intestinal HT-29 cells. [(57)]

### 3.3 Modulation of immune system

Probiotics should be able to stimulate as well as regulate a several aspects of the natural and the acquired immune response. Consumption of specific strains of probiotics has also been shown to enhance the immune response to natural infections and systematic or oral immunization. Probiotics confer immunological protection against enteropathogens by stimulating the cytokine production and macrophages; Enhancing the specific antibody response to the pathogens.[(58, 59)]

## 4. Lactic acid bacteria

Lactic acid bacteria (LAB) constitute of a group of gram-positive bacteria, non-spore forming cocci or rods, low G + C%, which produce lactic acid widely used in the food fermentation industry and are generally recognized as safe (GRAS) microorganisms. This group of bacteria has advanced acid tolerance and survive at < pH 5 giving them a competitive advantage over another microorganism.

LAB belong to the order *Lactobacillales*. These genera are: *Lactobacillus*, *Leuconostoc, Carnobacterium*, *Enterococcus*, *Oenococcus, Tetragenococcus, Vagococcus, Pediococcus, Lactococcus, Streptococcus, Carnobacterium, Enterococcus, Oenococcus, Tetragenococcus* and *Vagococcus*. LAB in Actinobacteria phylum only include species of *Bifidobacterium* genus. Currently, the most dominant LAB application is used as starter cultures for fermented food production, where they give in the flavor, texture and nutritional value of fermented foods. [(60, 61)]

LAB with probiotic properties are generally intestinal flora with beneficial functions in the ecology of the human digestive tract. [(62)] Various reports have shown that

traditional fermented foods are rich in LAB with probiotic characteristics.[63, 64] Health benefits of probiotic LAB are summarized in Table 3.

Table 3 Lactic acid bacteria derived probiotics and human health [64]

| Lactic Acid Bacteria | Effects on human health |
|---|---|
| *Lactobacillus acidophilus* | Secretes lactic acid which reduces the pH of the gut and inhibits the development of pathogens (*Salmonella* spp, *E.coli*). |
| *Lactobacillus johnsonii* | Effective in inhibition of *H. pylori* and against inflammation |
| *Lactobacillus plantarum* | Produces short-chain fatty acids that block the generation of carcinogenic agents by reducing enzyme activities |
| *Lactobacillus fermentum* | Effective in restoration of a normal microflora. Effective against bacterial vaginosis flora |
| *Lactobacillus reuteri* | Reduces the duration of diarrhea |
| *Enterococcus faecium* | Can reduce blood cholesterol leading to decreased blood pressure |

**5. Gut microbiota**

The human microbiome is composed of numerous microorganisms: bacteria, archaea, viruses and fungi that reside in and on our bodies. They have enormous potential to impact body physiology, both in health and in disease. The intestine is the organ with the most living microorganisms, consisting of $4 \times 10^{14}$ bacteria distributed across the entire gut.[65] The gut of humans contains trillions of bacterial cells from 500 to

1000 different bacterial species.[66, 67] Although bacteria with < 50 phyla described to date, the healthy gut microbiota is predominated only of them: phyla *Firmicutes* and *Bacteroidetes*, whereas *Proteobacteria*, *Verrucomicrobia, Actinobacteria*, *Fusobacteria*, and *Cyanobacteria* are present in minor proportions (Fig. 2, A and B).[68, 69] The number of bacterial cells immediate in gut shows a continuum that goes from $10^1$ to $10^3$ bacteria per gram of contents in the stomach and duodenum, progressing to $10^4$ to $10^7$ bacteria per gram in the jejunum and ileum and culminating in $10^{11}$ to $10^{12}$ cells per gram in the colon (Fig. 2A).[70]

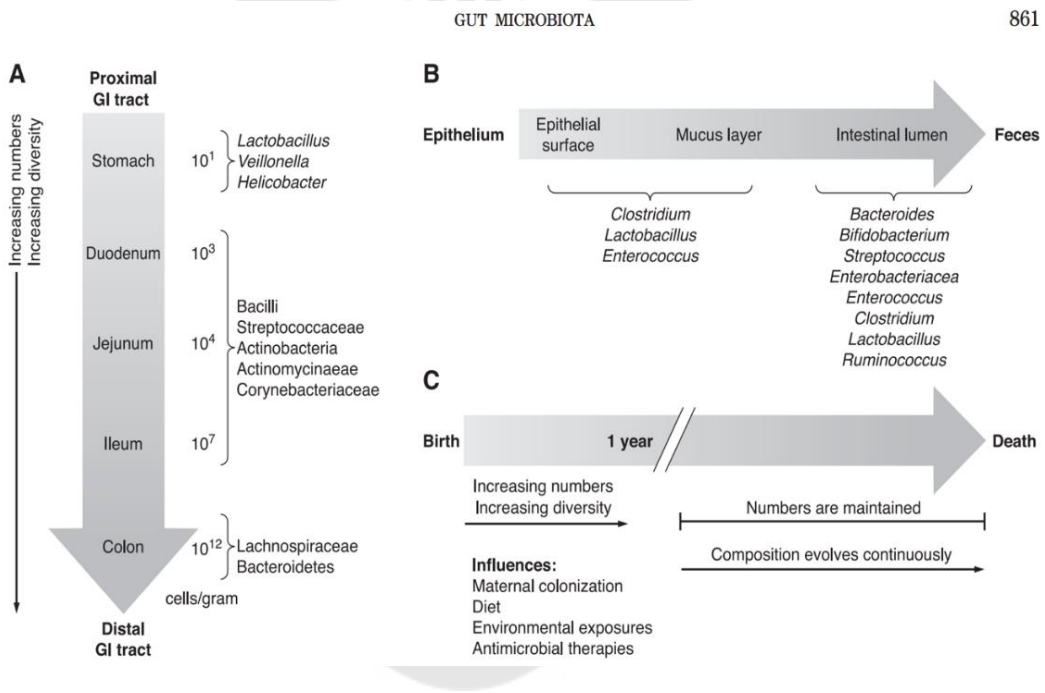



Figure 2 Spatial and temporal aspects of intestinal microbiota composition.

A: variations in microbial numbers and composition across the length of the gastrointestinal tract. B: longitudinal variations in microbial composition in the intestine. C: temporal aspects of microbiota establishment and maintenance and factors influencing microbial composition [70]

Gut microbiota has several important functions such as performing a barrier effect, protection against invasive bacterial strains and modulate immune system. Behavior dietary and lifestyle associated chronic diseases like hypertension, type 2 diabetes and Inflammatory bowel disease (IBD) are very concern worldwide.[71]

Short chain fatty acids (SCFA) are the primary end-products of fermentation of non-digestible carbohydrates (NDC) by gut microbiota. The synthesis of SCFA such as butyrate, propionate and acetate, which are rich sources of energy for the host. The SCFAs also have several effects on metabolism. For example, propionate and acetate are important substrates for gluconeogenesis and lipogenesis. Propionate improves insulin sensitivity. [72] Therefore, the production of SCFAs by intestinal microbiota is an important source of energy from food as well as important intermediates in gut motility and fat storage control.[73]

Interaction between microorganisms and host are involved in controlling the microbiota in the stomach. Gut epithelial tissue is creating effective physical barriers to prevent the access of environmental pathogens and antigens into the host's internal environment, release of chemicals and cytokines that receive inflammatory and immune cells, involved in the control of these potentially harmful agents.

The causes of obesity are excess energy intake and sedentary lifestyle, which are an important influence of imbalance gut microbiota and changing microbial populations associated with obesity.[74] In humans and animals that are obese, the microbial population changes with increasing in the *Firmicutes* and reducing in the *Bacteroidetes*, cause adiposity through greater energy harvest.[75, 76] However, other data show that the change in microbial population are caused by eating foods promoting excessive weight gain. Other lifestyle factors, stress, has a critical on intestine activity which can alter gut microbiota profiles, including of the lower numbers of potentially beneficial *Lactobacillus*.

Probiotic and Prebiotic are diet processes / strategies used as food to promote host health by improving the composition of the intestine microbiota. Prebiotic is a nutrient that stimulates the proliferation and / or function of “beneficial" bacteria in the

colon including probiotics, microorganisms. Thus, conferring benefits upon host health. The consumption of probiotics, prebiotics and nutrients, beneficial molecules or microbes are designed to be beneficial to the body by increasing the number of beneficial microorganisms or products they have with the intestines.[77] Some probiotics are known to reduce lactose intolerance. The stimulation of growth of *Bifidobacterium* in the colonic microbiota type has been shown to be inulin-type fructans (prebiotic) in both *in vitro* and in vivo. In addition, eating with probiotics and prebiotics also reduces the risk of disease and promotes health host.

## 6. Cholesterol

Cholesterol is compound of the sterol type found in most body tissues, including the blood and the nerves. Cholesterol and its derivatives are important constituents of cell membranes and precursors of other steroid compounds. The cholesterol in blood comes from two sources: the foods and liver.[78] But too much cholesterol in blood causes the risk of coronary heart disease.[79]

### 6.1 Lipoproteins

Lipoproteins are composed of an outer water-soluble surface and an inner water-insoluble core. The outer portion comprises phospholipid, protein and cholesterol, with triglyceride and cholesterol ester (a cholesterol molecule linked to a fatty acid) forming the core. The main role of lipoprotein particles is to transport fats such as triglycerides and cholesterol in the blood between the organs of the body.[80] Lipoproteins are divided into four main groups; each group has different cholesterol and triglycerides. They are classified according to the density, the lower the density of lipoprotein, the greater the amount of fat contained within. Fig. 3 There are five major types of lipoproteins; chylomicrons, very low-density lipoprotein (VLDL), intermediate-density lipoprotein (IDL), low-density lipoprotein (LDL) and high-density lipoprotein (HDL).[81]

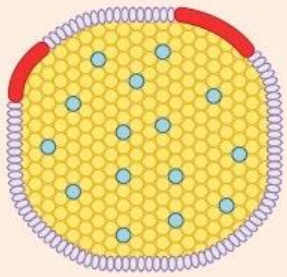
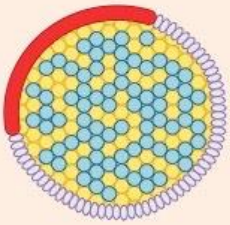
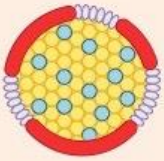
| | Chylomicron | LDL | HDL |
|---|---|---|---|
| **Diagram** |  |  |  |
| Triacylglycerol<br>Cholesterol<br>Phospholipid<br>Apoprotein | | | |
| **% Lipid** | | | |
| • Triglyceride | **98** | 15 | 10 |
| • Cholesterol | ~1 | **60** | 30 |
| • Phospholipid | ~1 | 25 | 60 |
| **% Protein** | | | |
| | 2 | 20 | **50** |

Figure 3 Major Types of Lipoproteins

Source: https://ib.bioninja.com.au/standard-level/topic-2-molecular-biology/23-carbohydrates-and-lipids/lipoproteins.html

### 6.1.1 Chylomicrons

Chylomicrons, the least dense of the lipoproteins are formed in the intestinal cell walls from dietary fat. Their main task is to carry triglycerides from the intestine to the tissues where they are needed as a source of energy. The blood circulation triglycerides is excreted from the chylomicrons via the action of lipoprotein lipase (LPL), an enzyme present in the capillaries of various tissues. If there are chylomicrons in large quantities, such as after eating fatty foods, chylomicrons cause the plasma to appear milky.[82, 83]

### 6.1.2 Very low-density lipoproteins (VLDL)

VLDLs are synthesized in the liver. They work like chylomicrons to distribute triglycerides to target sites such as fat tissue and skeletal muscle. They work like chylomicrons to distribute triglycerides to target sites such as fat tissue and skeletal muscle as it is used in storage and energy. Triglycerides are removed from the circulation just like chylomicrons. VLDL levels in high plasma are found in the group

hypertriglyceridemia, diabetes, underactive thyroid, and in people with high alcohol content.[84]

#### 6.1.3 Low density lipoproteins (LDL)

LDLs are cholesterol exuberant particles. approximately 70 % of cholesterol in plasma occurs in this form. LDLs are highly involved in the transport of cholesterol produced in the liver to tissues. Cholesterol Uptake in the cell occurs when the lipoprotein binds to the LDL receptor on surface. LDL cells are introduced into the cells and broken down into cholesterol and free amino acids.[85]

#### 6.1.4 High density lipoproteins (HDL)

HDL are consisting of 50 % protein, with phospholipid and cholesterol. HDL is commonly familiar as the 'good' cholesterol. The function of HDL is to transport excess cholesterol from the tissues (Including blood vessels) to the liver to eliminate. Many studies show that low HDL cholesterol levels are high risk for coronary heart disease. [86]

### 6.2 High cholesterol

Hypercholesterolemia is the term to indicate a high cholesterol serum level. When body have high cholesterol may develop fat deposits in blood vessels causing heart disease and increases in the risk of a heart attack. The World Health Organization (WHO) reprised that cardiovascular disease is the leading cause of death by 46% worldwide. [87]

## 7. Bile

Bile is a fluid, essential for intestinal digestion and absorption of lipids. Bile is synthesized by the liver. In addition, bile also has important properties for eliminating environmental toxins, carcinogens, drugs and their carcinogens.

### 7.1 Bile salt synthesis

Bile is produced by the liver from cholesterol, a precursor in the synthesis. Bile then is excreted into the gallbladder where it is concentrated or is delivered directly to the small intestine. Cholesterol, ingested as part of the diet or derived from hepatic

synthesis is converted into the bile acids cholic and chenodeoxycholic acids, which are then conjugated to an amino acid (glycine or taurine) to yield the conjugated form that is actively secreted into canaliculi.[88] The most universal bile acids in human bile are chenodeoxycholic acid (45%) and cholic acid (31%). These are quoted to as the primary bile acids. Before the primary bile acids are secreted into the canalicular lumen they are conjugated via an amide bond at the terminal carboxyl group with either of the amino acids glycine or taurine. These conjugation reactions produce glycoconjugates and tauroconjugates, respectively. This conjugation process enhanced the amphipathic nature of the bile acids making them more easily secretable as well as less cytotoxic. The conjugated bile acids are the major solutes in human bile. (Fig. 4)

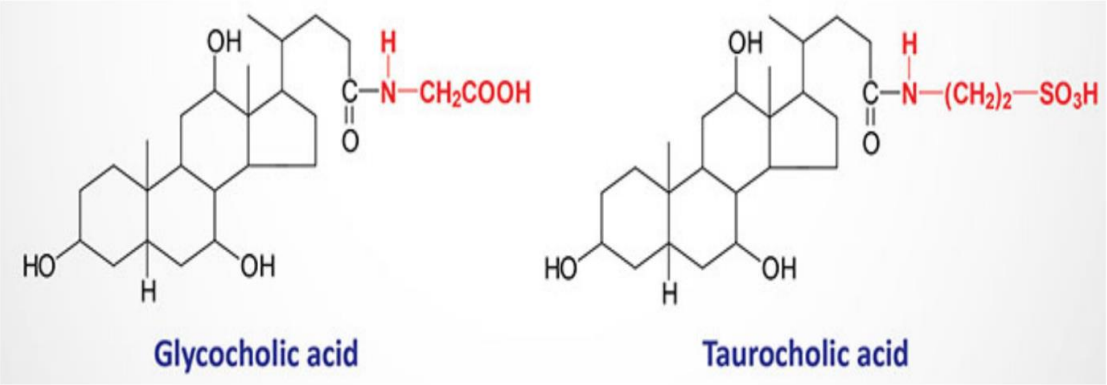



Figure 4 Structure of the conjugated cholic acids [89]

### 7.2 Roles of bile acids

Bile contains bile acids, which important for digestion and absorption of fats and fat-soluble vitamins in the small intestine. In addition, bile also has important properties including antimicrobial activity, primarily through the dissolution of bacterial membranes.[21]

**8. Probiotic bacteria as cholesterol-lowering**

High levels of lipids in blood are one major risk factors for cardiovascular diseases, type 2 diabetes mellitus, and metabolic syndrome [90] Drug treatment Hypocholesterolemia disadvantage is expensive and can have side effects. Risk factors the development of cardiovascular diseases are genetic factors and a sedentary lifestyle, environmental factors, including an increased intake of high-fat diets.[91] Recently, there has been increased attention for the function of the gut microbiota in the development of metabolic syndrome and its associated complications. Evidence studies suggests that probiotics can reduce serum cholesterol levels. But it only happens when they survive gastrointestinal conditions. This effect of lowering cholesterol levels initiates at the intestinal, makes it interested probiotics properties important in gut. [92]

**8.1 Bile salt hydrolase-active probiotic bacteria**

The potentiality of probiotic strains to hydrolyze bile salts has often been included among the criteria for probiotic strain selection and characterized by bile salt hydrolases (BSHs). *Clostridium*, and *Bacteroides* spp. *Lactobacilli* and *bifidobacteria* are most commonly used as probiotic, while *Bacteroides*, *Clostridium*, and *Enterococcus* spp. are also commensal bacteria inhabitants of the gastrointestinal tract. Overall, the data strongly support the hypothesis that microbial BSHs function in the detoxification of bile salts and in doing so increase the intestinal survival and persistence of producing strains. [93] Most probiotic strains with BSH activity are food grade bacteria that belong to the genera of *Lactobacillus*, *Bifidobacterium* and *Lactococcus*. The BSH gene has been found in most intestine microflora. However, strain identification together with BSH activity assessments, viability, resistance towards acid and bile, adherence to the intestinal epithelium, colonization of gut and hypocholesterolemia property can be some of the criteria for identifying the probiotics for industrial production of human food. [94]

### 8.2 Characteristics of bile salt hydrolase enzyme

BSH is a pharmacologically important enzyme. They belong to the Cholylglycine Hydrolase (CGH) family of the Ntn-hydrolase superfamily.[95] BSH is widely distributed in microorganism of mammal's digestive tract. BSH enzyme acts on the amide bond and catalyses the de-conjugation of tauro- or glyco-conjugated bile acids.[23] (FIG. 5.)

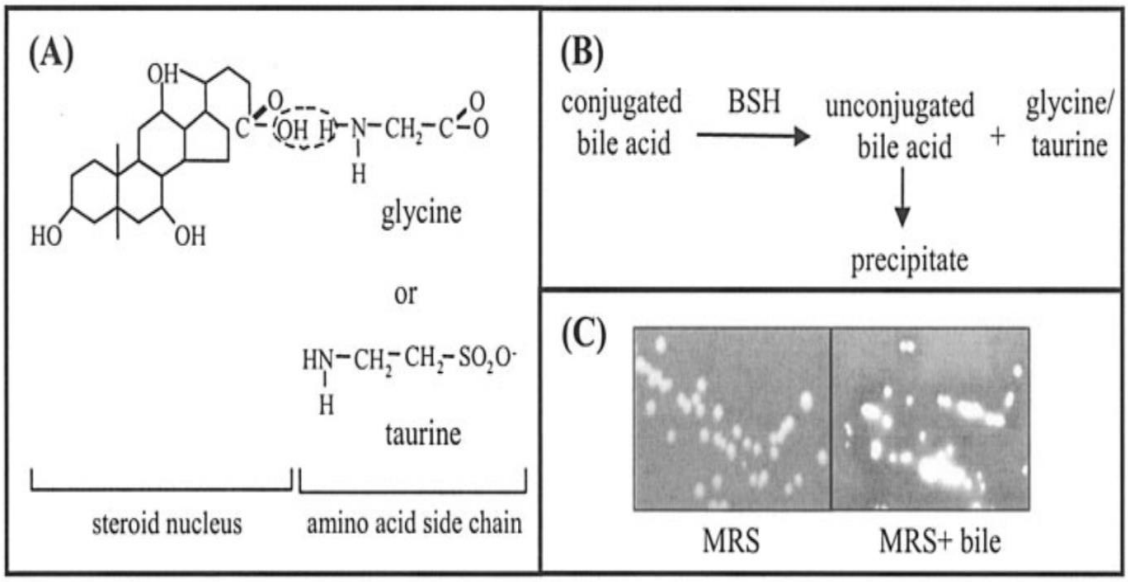



Figure 5 (A) Chemical structure of bile acids.

Primary bile acids are synthesized in the liver from cholesterol and are conjugated with either glycine or taurine prior to secretion. The carboxyl group of the bile acid and the amino group of the amino acid are linked by an amide bond. (B) Reaction catalyzed by BSH enzymes. BSHs cleave the peptide linkage of bile acids, which results in removal of the amino acid group from the steroid core. The resulting unconjugated bile acids precipitate at low ph. (C) Detection of BSH activity.[96]

### 8.3 Impact of BSH activity on the host

#### 8.3.1 Cholesterol lowering

Hypercholesterolemia or high blood cholesterol levels are a major risk factor for developing coronary heart disease, and even with such medication's agents

are available to treat this condition (such as statins or sequestrates), they are expensive or may have adverse side effects. [97] Oral administration of probiotics has been shown to significantly recede cholesterol levels 22 to 33% or prevent high cholesterol levels in mice fed high-fat-diet.[27] Deconjugated bile salts are few efficiently reabsorbed than conjugated bile salts, which results in the excretion of free bile acids in feces. Also, free bile salts are less capable in the solubilization and absorption of lipids in the gut. Therefore, deconjugation of bile salts causing de novo bile salt synthesis from cholesterol is used to maintain bile acid homeostasis, which will result decrease cholesterol in blood.

Table 4 summarizes findings for cholesterol lowering effects of probiotics [98, 99]

| | Probiotic organism | Experimental system | Major findings |
|---|---|---|---|
| *In vivo* | Yogurt (unknown) | Human | Reduced total cholesterol and LDL |
| *In vivo* | Fortified buffalo milk-yogurts with *B. longum* | Rat | Reduced total cholesterol, LDL-cholesterol and triglyceride |
| *In vivo* | *L. plantarum* | Mice | Reduced blood cholesterol Decreased triglycerides |
| *In vivo* | *L. plantarum* | Rat | Decreased total cholesterol and LDL-cholesterol |
| *In vivo* | *L. plantarum* | Culture media | Cholesterol assimilation |
| *In vivo* | *L. fermentum* | Culture media | BSH activity |

Table 4 (Continued)

| | Probiotic organism | Experimental system | Major findings |
|---|---|---|---|
| *In vivo* | *L. plantarum* | Rat | Decreased LDL, VLDL, and increased HDL with decrease in deposition of cholesterol and triglyceride in liver and aorta |
| *In vivo* | *L. acidophilus* | Culture media | Assimilation of cholesterol<br>Attachment of cholesterol onto cell surface |
| *In vivo* | *L. acidophilus* | | Deconjugation of bile salt<br>Bile salt hydrolase activity |
| *In vivo* | *L. casei* | | Deconjugation of bile salt<br>Bile salt hydrolase activity |
| *In vivo* | *L. bulgaricus* | | Attachment of cholesterol onto cell surface |
| *In vivo* | *L. reuteri*<br>*L. fermentum*<br>*L. acidophilus*<br>*L. plantarum* | Culture media | Cholesterol assimilation |

**9. Probiotics in food products**

Probiotics consumption through food products is one of the most popular approach at today. Most probiotic foods products are categorized as functional foods and are an important part of the diet. Demand for probiotic functional foods is growing rapidly due to increased consumer awareness. It has been estimated that probiotic

foods comprise between 60% and 70% of the total functional food market.[100, 101] Significant success has been achieved during the past few decades in development of dairy products containing probiotic bacteria, such as fermented milks, ice cream, various types of cheese, baby food, milk powder, frozen dairy desserts, whey-based beverages, sour cream, butter milk, normal and flavored liquid milk. [102]

**9.1 Probiotic dairy products**

In recent years, there has been a trend for more consumers to pay attention to health and seek foods that have additional functional properties for their nutrition. Probiotic dairy products are considered to have functional properties because the probiotic bacteria added to health benefit on the host such as modification of the immune system, reduction in cholesterol, alleviation from lactose intolerance and treatment and relief of diarrhea.[103, 104] In addition, airy probiotics can be as a food consumed by both children and adults. For example, four-week consumption of *Lactobacillus paracasei* subsp. paracasei LC01 in healthy adults resulted in reduced faecal *Escherichia coli* and ammonia, and increases in *Lactobacillus*, *Bifidobacterium*, and *Roseburia* intestinalis and acetic and butyric acid.[105]

**9.2 Probiotic fermented milk (yoghurt)**

Yogurt is a fermented milk product that is fermented by adding a specific lactic acid culture to the milk. The cultures probiotics used to make yogurt are *Lactobacillus bulgaricus*, *Streptococcus thermophilus* and *Lactobacillus casei*. [106] Yoghurt with probiotic properties help to maintain the balance of bacteria needed to boost the immune system and promote a healthy digestive tract. The international standard requires any cultured products sold with health claims to contain at least $10^7$ CFU / g of viable probiotics at the time of consumption.[107]

**9.3 Beneficial health effects of probiotic products**

Probiotics give a number of health benefits generally through maintenance of normal intestinal microflora, protection against gastrointestinal pathogens, reduction of serum cholesterol level and blood pressure, improved utilization of nutrients and improved nutritional value of food [108] (Fig. 6)

**9.3.1 Cardiovascular diseases**

There is preliminary evidence that use of probiotic *lactobacilli* and metabolic by products potentially confer benefits to the heart, including prevention and therapy of various ischemic heart syndromes and lowering serum cholesterol by BSH-active properties.[109]

While the Consultation believes these findings to be important, more research and particularly human studies are required before it can be ascertained that probiotics confer health benefits to the cardiovascular system.

**9.3.2 Lactose intolerance**

Lactose intolerance causes abdominal discomfort affecting about 70% of the world's population. Probiotic microorganism *L. acidophilus* bacteria have been clinically shown to relieve lactose intolerance.[110]

**9.3.3 Supports healthy digestion**

Healthy bacteria that are added to fermented milk help to improve the microflora in the gut, which is responsible for digestion and a healthy digestive tract. These active cultures may help with certain gastrointestinal conditions, including colon cancer, IBS, constipation, diarrhea and lactose intolerance.[79, 111]

**9.3.4 Hypocholesterolemia properties**

There is preliminary evidence that the live probiotics in yogurt, including *Lactobacillus acidophilus*, decrease cholesterol levels, with just one 200-milliliter (seven ounces) serving per day. In a controlled clinical study, researchers witnessed a 2.4 percent reduction in serum cholesterol. They believe that regular probiotic yogurt consumption has the potential to reduce the risk of coronary heart disease by 6 to 10 percent. [112]

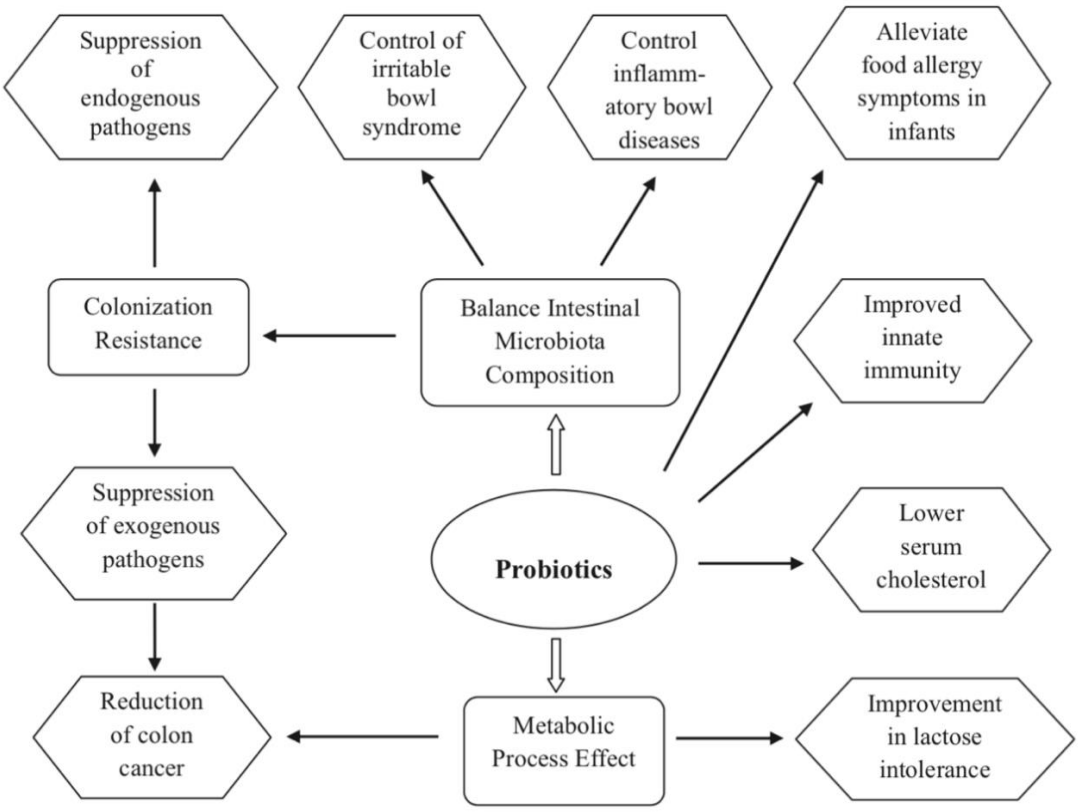



Figure 6 Probiotics consumption and health benefits. [107]

**9.4 Commercially used microorganisms for probiotic foods**

Probiotics used in commercial foods are mostly genera *Lactobacillus* and *Bifidobacterium* (Tables 4). The primary reason being both these genera have a long history of safe use and are considered as GRAS. *Lactobacillus* and *Bifidobacterium* species are inhabitants in the human intestine (*Lactobacillus* in the small intestine and *Bifidobacterium* in the large intestine). [113]

Table 5 Some industries strains probiotic commercial used by various industries [114]

| Source / product | Strain |
|---|---|
| Chr. Hansen | *L. acidophilus* LA1/LA5 |
| | *L. delbrueckii* ssp. bulgaricus Lb12 |
| | *L. paracasei* CRL431 |
| | *B. animalis* ssp. lactis Bb12 |
| Danisco | *L. acidophilus* NCFMs |
| | *L. acidophilus* La |
| | *L. paracasei* Lpc |
| DSM Food Specialties | *L. acidophilus* LAFTIs L10 |
| | *B. lactis* LAFTIs B94 |
| | *L. paracasei* LAFTIs L26 |
| Snow Brand Milk | *L. acidophilus* SBT-20621 Products Co. Ltd. *B. longum* SBT-29281 |
| Biogaia | *B. long um* BB536 |
| *Foneterra Probi AB Danone* | *L. rhamnosus* HN001 (DR20 |
| | *B. lactis* HN019 (DR10) |
| *Institute Rosell* | *L. rhamnosus R0011* |
| | *L. acidophilus R0052* |
| Yakult | *L. casei* Shirota |
| | *B. breve* strain Yaku |
| Morinaga Milk Industry Co. Ltd. Lacteol | *L. acidophilus* LB |
| Essum AB | *L. plantarum* 29 |

### 9.5 Doses of probiotics

The Food and Drug Administration (FDA or USFDA) has also suggested that the minimum probiotic count in a probiotic food should be at least $10^6$ CFU $ml^{-1}$.[115] Depending on the amount ingested and taking into account the effect of storage on probiotic viability, a daily intake of $10^8$– $10^9$ probiotic microorganisms is essential to achieve probiotic action in the human organism. It is also been stated to probiotic products should be consumed regularly approximately 100 grams of probiotics per day to deliver $10^9$ live cells in the intestine.[116]

### 9.6 Development of probiotic foods

Over the past few decades more than 500 probiotic food products have been introduced in the global market. [117] Probiotic culture used in food product, therefore, should not adversely affect product quality or sensory properties. Most of the culture preparation is commercially available in highly concentrated form and the use of starter cell concentrates designated as either Direct Vat Set (DVS) or Direct Vat Inoculation (DVI), such as high concentration frozen or dry powdered form. Frozen cultures contain more than $10^{10}$ CFU $g^{-1}$, whereas freeze-dried cultures typically contain more than $10^{11}$ CFU $g^{-1}$. The packaging materials used and the storage conditions of the product storage are important to the quality of the probiotic product. [4] The technological properties associated with the incorporation of probiotic strains into food products are presented in Fig. 7

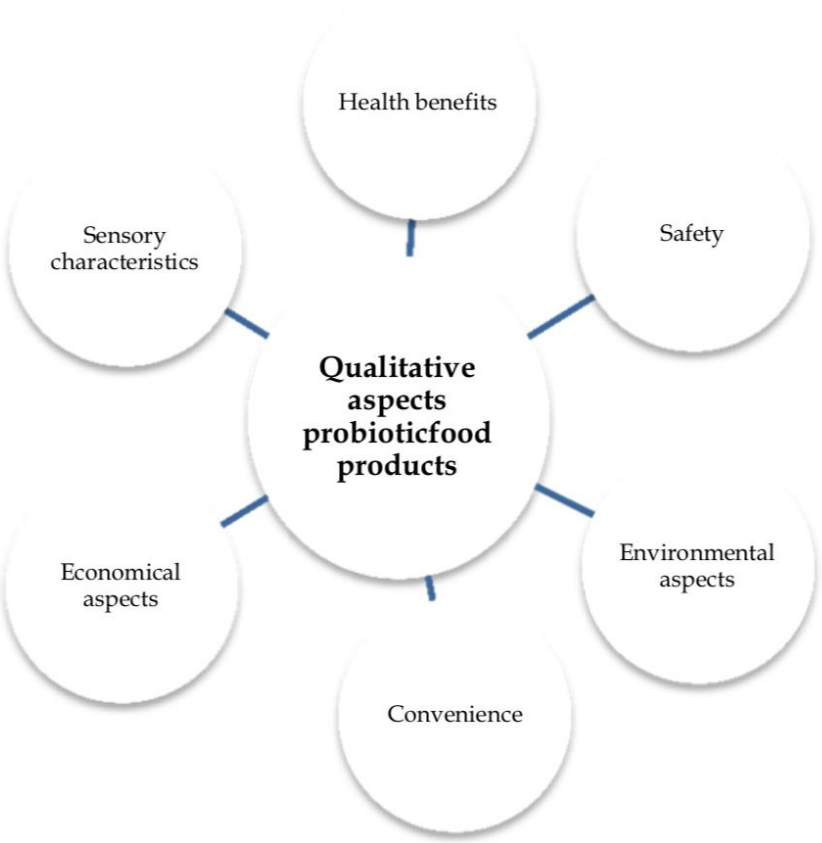



Figure 7 Qualitative aspects of probiotic food products.[118]

### 9.7 Survival of probiotics during processing and storage

The pharmaceutical efficacy of probiotic food products depends on the number of live and active cells per in grams or milliliters of the food product at the moment of consume. [119] Therefore, it is important to ensure high probiotic survival rates during production as well as shelf life to maintain consumer confidence in probiotics. There are many factors that influence the viability of probiotics microorganisms in food products during production, processing and storage include food parameters: pH, titratable acidity, molecular oxygen, and water activity, presence of salt, sugar and chemicals like hydrogen peroxide, bacteriocins, artificial flavoring and coloring agents. (Fig. 8) [120]

### 9.7.1 Fermentation conditions

The temperature used during fermentation is one of the major factors affecting the viability of probiotics and other qualitative parameters of probiotic fermented products. The optimal temperature for growth of probiotics is in the range of 37-43°C. [121] Exposure to oxygen during fermentation plays an important role in reducing the viability of oxygen-sensitive bacteria. Various methods are used to reduce oxygen during fermentation. The most important thing is to make the fermentation under the vacuum. [122]

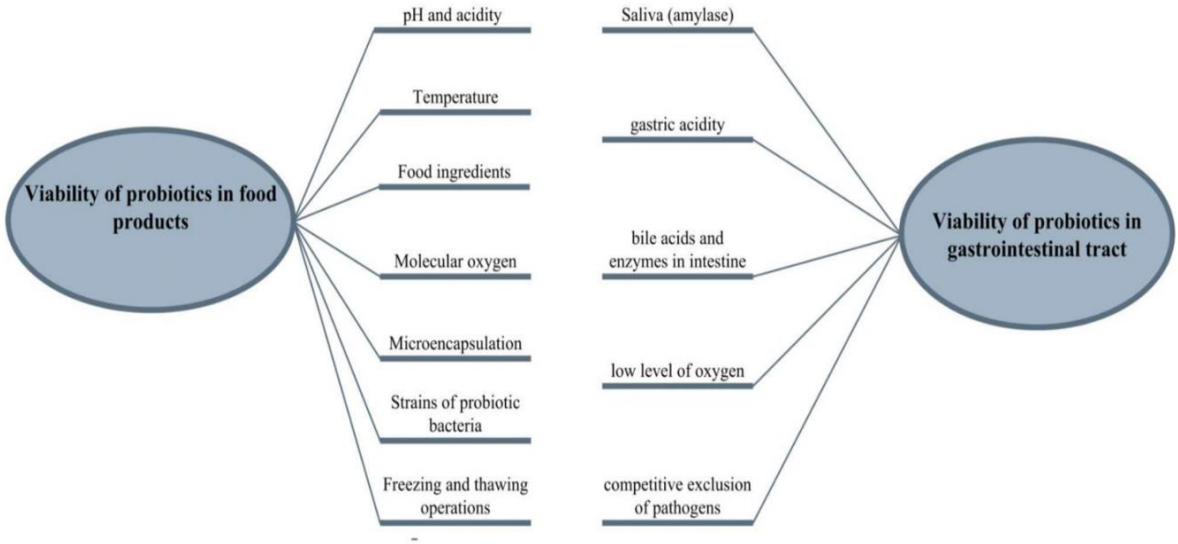



Figure 8 Main factors affecting the viability of probiotic food products and during delivery through gastrointestinal tract. [120]

### 9.8 Storage

Probiotic supplements should be stored at 4 to 5°C to maintain viability of the microorganisms and should be used before the expiry date. Probiotic products must be stored in a cool place, otherwise they will not stay viable. Some probiotics products, which may be stable products as defined to their manufacturers and stored and shipped requirements must be met. [123]

# CHAPTER 3
# METHODOLOGY

## Materials

1. De Man Rogosa Shape (MRS) media (Oxoid, Basingstoke, Hampshire, UK)
2. Calcium carbonate ($CaCo_3$)
3. Glycerine (Sigma, USA)
4. Skim milk (Difco, USA)
5. Anaerobic gas package (MGC, japan)
6. Anaerobic jar (Mitsubishi, Japan)
7. Light microscope (Nikon, Japan)
8. pH meter (Thermo Scientific, USA)
9. Incubator (Selecta, Spain)
10. Autoclave (Selecta, Spain)
11. Laminar flow hood (Nuaire, USA)
12. Centrifuge (Sartorius Stedim, Germany)
13. Spectrophotometer UV (Shimadzu, Japan)
14. Sodium salt of taurodeoxycholic acid (TDCA) (Sigma, USA)
15. Calcium chloride ($CaCl_2$) (Merck, Germany)
16. Carbohydrates (Merck, Germany)
17. L- arabinose, cellobiose, D-galactose,gluconate, melibiose, α-methyl-D-glucoside, Rhamnose, salicin, trehalose, sucrose, Ribosemaltose
19. Xylene
20. Hydrochloric acid (HCL) (Merck, Germany)
21. Potassium phosphate dibasic ($K_2HPO_4$) (Merck, Germany)
22. PCR Authorized thermal Cycler (Eppendrof,

Germany)

23. PCR DNA fragment extraction kit (Geneaid Biotech, Taiwan)

24. Gel electrophoresis chamber MiniRun GE-100 (HandzhouBioer technology, China)

25. Ox gall (sigma, USA)

**Methods**

**1. Isolation and selection of lactic acid bacteria**

Lactic acid bacteria were isolated from Thai fermented foods. Ten milliliters De Man Rogosa Shape (MRS) broth was used to enrich and cultivate 1 g of fresh samples and incubated at 37°C for 48 h under anaerobic condition using anaerobic jar. One loop full of broth culture was transferred and streaked on MRS agar plate containing 0.3% calcium carbonate (CaCO3). One single pure colony of lactic acid bacteria was selected by the presence of transparent halo-surrounding the colony. The isolates were firstly screened for catalase activity and Gram staining and was selected only those that are catalase-negative and Gram-positive. Glycerol stock (30% glycerol v/v) of pure cultures was maintained at -80°C for future studies. [124, 125]

**2. Screening of lactic acid bacteria for bile salt hydrolase activity**

Lactic acid bacteria were screened for bile salt hydrolase (BSH) activity by qualitative direct plate BSH assay. Ten microliters ($10^9$ CFU/ml) of overnight grown cultures in MRS broth was spotted onto sterile MRS agar plates supplemented with 0.5% sodium salt of taurodeoxycholic acid (TDCA, Sigma, USA) and 0.37 g/l of calcium chloride ($CaCl_2$). Plates were incubated anaerobically at 37°C for 72 hours. After incubation, plates were observed for the appearance of precipitation zones. The BSH activity was determined by the diameter of the precipitation zones. The assay was performed in duplicate.[126-128]

**3. Genotypic characteristics by 16S rRNA gene sequencing**

Lactic acid bacteria grown at 37°C for 24 h on MRS agar was used for 16S rRNA gene sequences. The 16S rRNA gene sequences coding region was amplified by PCR in a PCR thermal cycler. The sequences of the PCR products using the prokaryotic 16S ribosomal DNA universal primers 27F(5'AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') were purified using a Geneaid Gel/PCR DNA fragment Extract Kit ( Geneaid Biotech, Bade City, Taiwan). The sequenced analysis of PCR products by Macrogen, Korea was done using universal primers. The similarity of 16S rRNA gene sequences was determined using BLAST software compared to EzTaxon-e database. Multiple alignments of sequence were performed by CLUSTAL X in BioEdit Sequence Alignment Editor Software. The phylogenetic tree was constructed by the neighbor-joining in the MEGA 7 software..[129]

**4. Phylogenetic analysis in molecular evolutionary genetics**

The 16S rRNA gene sequences obtained was added to publicly available bacterial 16S rRNA gene sequences were integrated to the database were an automatic alignment tool. Phylogenetic tree was generated by performing distance matrix analysis using the neighbor joining method. Database search and comparison was done the program MEGA version 7 software. The confidence values of individual branches in the phylogenetic tree were determined using the bootstrap analyses of based on 1000 replications[130]

**5. Acid and bile tolerance test**

**5.1. Acid tolerance**

Selected LAB isolates were cultivated into MRS broth at 37°C for 48 h under anaerobic condition. Each strain of $10^9$ CFU/ml was inoculated into MRS broth at various pH values 2.0, 3.0 and 4.0 adjusting with hydrochloric acid (1N HCL). The cultures were incubated at 37°c for 3 h under anaerobic condition using anaerobic jar. After incubation, 10-fold serial dilution was performed with the addition of phosphate

buffer (pH 7.2). One hundred microliters of each serial dilution ($10^{-4}$-$10^{-6}$) was transferred onto MRS agar plate. The spread plate technique was used and incubated at 37°C for 48 h under anaerobic condition using anaerobic jar. Total viable counts were determined after 3 h incubation and displayed by the log 10 of colonies grown on MRS agar. Unadjusted pH MRS broth (pH 6.5) was used as a control. All experiments were done in duplicate and two experiments. [131]

**5.2. Bile tolerance assay**

Selected LAB isolates were cultivated into MRS broth at 37°C for 48 h under anaerobic condition. Each strain containing $10^9$ CFU/ml was inoculated into MRS broth at various concentrations of bile salt (0.3 and 0.8%) using Ox gall. The cultures were incubated at 37°C for 3 h under anaerobic condition using anaerobic jar. After incubation, 10-fold serial dilution was performed with the addition of phosphate buffer (pH 7.2). One hundred microliters of each serial dilution ($10^{-4}$-$10^{-6}$) was transferred onto MRS agar plate. The spread plate technique was used and incubated at 37°C for 48 h under anaerobic condition using anaerobic jar. Total viable counts were determined after 3 h incubation and displayed by the log 10 of colonies grown on MRS agar. Unadjusted pH MRS broth (pH 6.5) was used as a control. All experiments were done in duplicate and two experiments. [131]

**6. Cell surface hydrophobicity**

Bacterial adhesion was determined to assess the adherence potential of microorganisms to surface hydrocarbons, which is a measure of adhesion to epithelial cells of the gut. Lactic acid bacteria were allowed to grow in MRS broth for 18 h and centrifuged. Pellets were washed twice with phosphate urea magnesium sulfate buffer. Pellets were re-suspended in buffer, vortex, and adjusted to absorbance 0.7–0.9 at 600 nm (A0). The cell suspension (3.0 ml) was mixed with 1 ml of hydrocarbon (xylene) and incubated at 37°C for 1 h for aqueous and organic phase separation.

The aqueous phase (1 ml) was carefully removed and absorbance was measured at 600 nm (A1). Percent hydrophobicity was measured by a decrease in absorbance and calculated using following formula.

$$\text{Percent hydrophobicity} = (1 - A1/A0) \times 100$$

The degree of a strain's hydrophobicity was assigned as hight hydrophobicity, moderate hydrophobicity and low hydrophobicity within percentage adhesion values equal >70%, 50–70% and <50% respectively [132]

**7. Hemolytic activity**

Lactic acid bacteria isolates were grown for 18 h in MRS medium at 37 °C, and then transferred onto Blood Agar Base plates containing 5 % (v/v) human blood. The plates were incubated for 24 h at 37 °C. The hemolytic reaction was recorded by observation of a clear zone of hydrolysis around the colonies (β-hemolysis), a partial hydrolysis and greening zone (α-hemolysis), or no zone of clearing around the colony (γ-hemolysis), which was considered negative. *Streptococcus pyogenes* was served as positive control. [133]

**8. Antibiotic resistance**

The antibiotic resistance patterns of probiotic LAB were determined by a disk diffusion method using the Kirby-Bauer technique. The antibiotic resistance of isolated LAB was assessed using antibiotic discs (Commercial discs from Hi media Laboratories Pvt. Ltd. Mumbai, India) on the surface of Mueller Hinton Agar (MHA) agar and the plates were kept at 40C for 1 h for diffusion, and then incubated at 37C for 24 h. Selected LAB isolates were cultivated into MRS broth at 37°C for 24 h under anaerobic condition. Each strain of selected lactic acid bacteria at the concentration of $10^8$ CFU /ml. MHA agar plates with a thickness of 4 ± 1 mm was evenly spreader with 10 ml of probiotic LAB (8.0 log CFU /ml) using a cotton sterile swab. Antibiotic discs were placed which included Chloramphenicol 30 μg, Tetracycline 30 μg, Nalidixic acid 30 μg,

Ampicillin 10 μg, Gentamicin 10 μg and Streptomycin 10 μg on the plates and incubated for 36 h at 37˚C. The diameter of the inhibition zone was measured with a Vernier caliper, and the antibiotic resistance was determined according to the Clinical & Laboratory Standards Institute (CLSI). [134]

**9. Antimicrobial activity against pathogens**

Twelve strains that are pathogenic to humans were used as pathogens to investigate the antagonistic activity of the selected LAB. They are *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 6538, *Vibrio cholera, Vibrio parahaemolyticus*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Shigella dysenteriae*, *salmonella typhi*, *Staphylococcus aureus* MRSA DSMT 20654, *Helicobacter pylori* and *Streptococcus pyogenes*, obtained from the culture collection of Department of Microbiology, Srinakharinwirot University, Thailand.

Each strain of selected LAB was grown in 10 ml MRS broth at 37 °C overnight, after which the cultures were centrifuged at 4000×g for 10 min at 4 °C for removal of bacterial cells. Part of the cell-free supernatants (CFS) was filtration (0.22 μm pore size; Millipore). An agar-well diffusion assay was used, aliquots of 50–60 μl of the sterile cell free supernatant were placed in 7 mm diameter wheels on Muller–Hinton-agar plates previously seeded with the respective test pathogens. After 24 h of incubation at 37 °C, the diameters of the zones of growth inhibition were measured. Inhibition zones more than 20 mm, 10–20 mm and less than 10 mm were reviewed as strong, intermediate and low inhibition, respectively. The test was performed twice, each in triplicate. [135]

**10. Fermented milk products using probiotics**

Fresh milk (fresh milk 90% (w/w) skim milk 10%) was heated at 80°C for 30 min and allowed to cool to 40°C. Milk was inoculated (3% v/v) with selected probiotic lactic acid bacteria of single culture and mixed of culture at a ratio of 1;1;1 the initial concentration of bacteria was $10^9$ CFU/ml. Incubated at 40°C until pH of the milk reaches 4.6. Changes of pH during fermentation was measured using a digital

pH-meter. Quality parameters of fermented milk during storage at 5°C was evaluated every 7 days for 28 days. The parameters include the viability, pH, Acid and bile tolerance, examine the existence of BSH activity in fermented milk, texture, syneresis, rheological properties and microstructure.

**11. The quality parameters of probiotic lactic acid bacteria in fermented milk**

**11.1 Measurement of viability**

After incubation, 10-fold serial dilution was performed with the addition of phosphate buffer (pH 7.2). One hundred microliters of each serial dilution ($10^{-5}$-$10^{-9}$) was transferred onto MRS agar plate. The spread plate technique was used and incubated at 37°C for 48 h under anaerobic condition using anaerobic jar. Total viability was counted and displayed as cell numbers in the log 10 of colonies grown on MRS agar.

**11.2 Measurement of pH**

The pH values of the fermented milk products were determined using a pH meter after calibration with fresh pH 4.0, 7.0 and 10.0 standard buffers.

**11.3 Measurement of Acid and bile tolerance test**

**11.3.1 Bile tolerance assay of selected LAB in fermented milk**

One hundred microliters of fermented milk were transferred onto MRS broth at various concentrations of bile salt (0.3 and 0.8%) using Ox gall. The cultures were incubated at 37°C for 3 h under anaerobic condition using anaerobic jar. After incubation, 10-fold serial dilution was performed with the addition of phosphate buffer (pH 7.2). One hundred microliters of each serial dilution ($10^{-4}$-$10^{-6}$) was transferred onto MRS agar plate. The spread plate technique was used and incubated at 37°C for 48 h under anaerobic condition using anaerobic jar. Total viable counts were determined after 3 h incubation and displayed by the log 10 of colonies grown on MRS agar. Unadjusted pH MRS broth (pH 6.5) was used as a control. All experiments were done in duplicate and two experiments

**11.3.2 Acid tolerance of selected LAB in fermented milk**

One hundred microliters of fermented milk were transferred onto MRS broth at various pH values 2.0, and 3.0 adjusting with hydrochloric acid (1N HCL). The cultures were incubated at 37°C for 3 h under anaerobic condition using anaerobic jar after incubation, 10-fold serial dilution was performed with addition of phosphate buffer (pH 7.2). One hundred microliters of each serial dilution ($10^{-4}$-$10^{-6}$) were transferred onto MRS agar plate. Spread plate technique was used and incubated at 37°C for 48 h under anaerobic condition using anaerobic jar. Total viable counts were determined after 3 h incubation and displayed by the log 10 of colonies grown on MRS agar. Unadjusted pH MRS broth (pH 6.5) was used as control. All experiments were done in duplicate and two experiments.

**11.4 Measurement of the existence of BSH activity in fermented milk.**

One hundred microliters of fermented milk were transferred onto MRS broth containing various pH values 2.0, and 3.0 adjusting with hydrochloric acid (1N HCL).

**11.5 Measurement of texture**

After inoculation, the samples of fermented milk products were poured into plastic containers of 52 mm in diameter to a height of 50 mm was reached. The texture of fermented milk was determined using a compression test carried out with a TA. XTplus Texture Analyser (Stable Micro Systems, Surrey, UK). A 20 mm acrylic cylinder probe was used. The test speed is fixed at 1 mm/s and the penetration depth is 10 mm. Firmness was expressed as gram (g), which is a peak force of compression. The texture analysis experiments were repeated three times on different dates.

**11.6 Measurement of syneresis**

Fermented milk (about 30 g) prepared in centrifuge tubes were centrifuged at 680 x g for 10 min at 4°C. Percent (%) syneresis is calculated as shown in equation..[136]

% Syneresis = Weight of whey (g)/total weight of milk (g) ×100

**11.7 Measurement of rheological properties**

Dynamic oscillatory measurements were performed with a HAAKE RheoStress1Rheometer (Thermo Scientific, Waltham, MA, USA) using a plate and cone geometry with 1 mm gap at 4°C. Amplitude sweeps were carried out with strain ranging from 0.05 to 100% and a frequency of 1 Hz. Storage modulus (G') and tan delta (tan$\delta$) was obtained. [137]

**12. Statistical analysis**

Results are expressed as mean ± SD. Statistical significance ($P<0.05$) between groups was determined using One-Way Anova, Tukey's test, GraphPad Prism 8.0

# CHAPTER 4
# RESULTS

## 1. Isolation and selection of lactic acid bacteria

A total of 55 lactic acid bacteria were isolated from 10 Thai fermented foods. They were tested for catalase activity and Gram staining. All isolates were found to have the typical characteristics of lactic acid bacteria (LAB). They are Gram-positive, bacilli or cocci and catalase-negative. Fifty-five isolates of lactic acid bacteria are shown in table 6.

Table 6 Isolation of lactic acid bacteria

| Sample | Location | Isolate no. | Gram reaction | Catalase test | Lactic acid |
|---|---|---|---|---|---|
| Nham moo | Mahasarakham | MN | + | - | + |
| Nham moo | Mahasarakham | MN2 | + | - | + |
| Nham moo | Mahasarakham | MN3 | + | - | + |
| Nham moo | Mahasarakham | MN4 | + | - | + |
| Raw-sausages | Mahasarakham | M | + | - | + |
| Raw-sausages | Mahasarakham | M2 | + | - | + |
| Raw-sausages | Mahasarakham | M3 | + | - | + |
| Raw-sausages | Mahasarakham | M4 | + | - | + |
| Sour meat | Mahasarakham | SM | + | - | + |
| Sour meat | Mahasarakham | SM2 | + | - | + |
| Sour meat | Mahasarakham | SM3 | + | - | + |
| Pickled garlic | Mahasarakham | PG | + | - | + |
| Pickled garlic | Mahasarakham | PG2 | + | - | + |
| Pickled garlic | Mahasarakham | pG3 | + | - | + |
| Pickled garlic | Mahasarakham | PG4 | + | - | + |
| Pickled lettuce | Udonthani | PL | + | - | + |

Table 6 (Continued)

| Sample | Location | Isolate no. | Gram reaction | Catalase test | Lactic acid |
|---|---|---|---|---|---|
| Pickled lettuce | Udonthani | PL2 | + | - | + |
| Pickled lettuce | Udonthani | PL3 | + | - | + |
| Pickled bamboo | Udonthani | PB | + | - | + |
| Pickled bamboo | Udonthani | PB2 | + | - | + |
| Pickled bamboo | Udonthani | PB3 | + | - | + |
| Pickled bamboo | Udonthani | PB4 | + | - | + |
| Pickled bamboo | Bangkok | PB5 | + | - | + |
| Pickled bamboo | Bangkok | PB6 | + | - | + |
| Pickled bamboo | Bangkok | PB7 | + | - | + |
| Pickled bamboo | Bangkok | PB8 | + | - | + |
| Pickled ginger | Bangkok | PG | + | - | + |
| Pickled ginger | Bangkok | PG2 | + | - | + |
| Pickled ginger | Bangkok | PG3 | + | - | + |
| Pickled ginger | Bangkok | PG4 | + | - | + |
| Pickled ginger | Bangkok | PG5 | + | - | + |
| Pickled ginger | Bangkok | PG6 | + | - | + |
| Kimchi (Thai) | Bangkok | KC | + | - | + |
| Kimchi (Thai) | Bangkok | KC2 | + | - | + |
| Kimchi (Thai) | Bangkok | KC3 | + | - | + |
| Kimchi (Thai) | Bangkok | KC4 | + | - | + |
| Pickled fish | Udonthani | PF | + | - | + |
| Pickled fish | Udonthani | PF2 | + | - | + |
| Pickled fish | Udonthani | PF3 | + | - | + |
| Pickled fish | Udonthani | PF4 | + | - | + |
| Pickled fish | Udonthani | PF5 | + | - | + |

Table 6 (Continued)

| Sample | Location | Isolate no. | Gram reaction | Catalase test | Lactic acid |
|---|---|---|---|---|---|
| Pickled fish | Udonthani | PF6 | + | - | + |
| Pickled shrimp | Udonthani | PS | + | - | + |
| Pickled shrimp | Udonthani | PS2 | + | - | + |
| Pickled shrimp | Udonthani | PS3 | + | - | + |
| Pickled shrimp | Udonthani | PS4 | + | - | + |
| Pickled shrimp | Udonthani | PS5 | + | - | + |
| Preserved lemon | Bangkok | PL | + | - | + |
| Pickled shrimp | Bangkok | PL2 | + | - | + |
| Pickled shrimp | Bangkok | PL3 | + | - | + |
| Rice fermented | Udonthani | RF | + | - | + |
| Rice fermented | Udonthani | RF2 | + | - | + |
| Rice fermented | Udonthani | RF3 | + | - | + |
| Rice fermented | Udonthani | RF4 | + | - | + |
| Rice fermented | Udonthani | RF5 | + | - | + |

**2. Bile salt hydrolase activity**

Fifty-five isolates of lactic acid bacteria were screened for bile salt hydrolase (BSH) activity by qualitative direct plate BSH assay containing 0.5% sodium salt of taurodeoxycholic acid and 0.37 g/l of calcium chloride ($CaCl_2$) as substrate. After 72 h of incubation, plates were observed for the appearance of precipitation zones around colonies, which indicated that that added bile salt was deconjugated by the action of bacteria bile salt hydrolase. The result showed that 3 of 55 showed that 3 of 55 isolates, MN, MN2 and SM, possessed the strongest ability to produce BHS (Table 7). These lactic acid bacteria had ability to produce BSH enzyme. The deconjugation of BSH activity of LAB isolates and widespread amounts of deoxycholic acid precipitated

around active colonies and diffused into the surrounding medium were shown in Fig. 9-11. LAB with the strong ability to produce BSH were selected for next studies.

Table 7 Bile salt hydrolase of isolate lactic acid bacteria by qualitative direct plate BSH assay

| Isolate no. | Bile salt hydrolase activity |
|---|---|
| MN | +++ |
| MN1 | + |
| MN2 | +++ |
| MN3 | + |
| KC2 | + |
| KC4 | + |
| SM | +++ |
| SM1 | + |

+++, Strong bile salt hydrolase activity; ++, modulate bile salt hydrolase activity; +, weak bile salt hydrolase activity

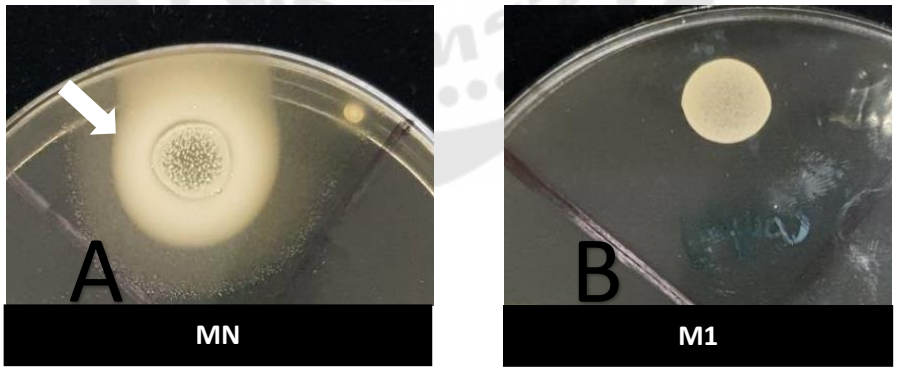



Figure 9. Bile salt hydrolase activities of isolate MN on MRS agar; precipitation zone was presented by white arrow.

A: Bile salt hydrolase activity of isolate MN on TDCA plate

B: Non-bile salt hydrolase plate

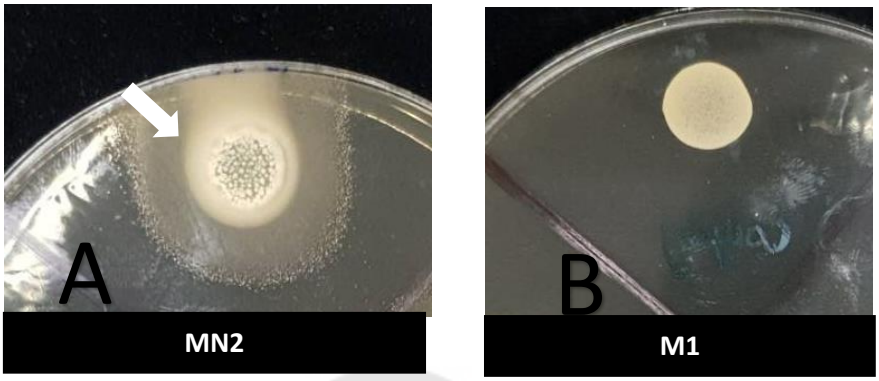



Figure 10. Bile salt hydrolase activities of isolate MN2 on MRS agar; precipitation zone was presented by white arrow.

A: Bile salt hydrolase activity of isolate MN2 on TDCA plate

B: Non-bile salt hydrolase plate

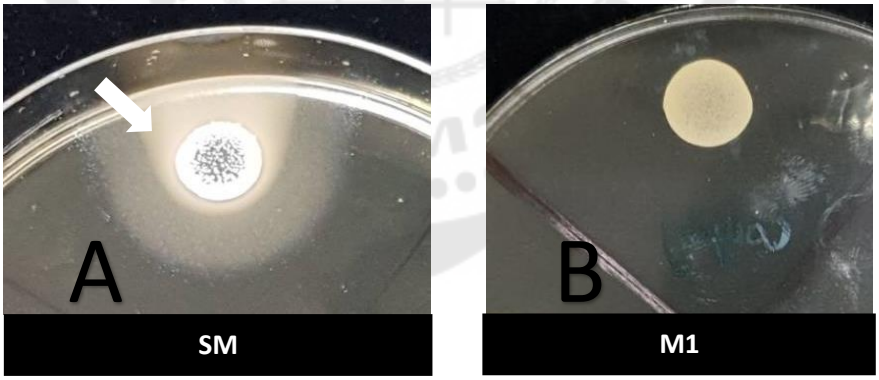



Figure 11. Bile salt hydrolase activities of isolate SM on MRS agar; precipitation zone was presented by white arrow.

A: Bile salt hydrolase activity of isolate SM on TDCA plate

B: Plate non-bile salt hydrolase

**3. Genotypic characteristics by 16S rRNA gene sequencing**

Three selected LABS with strong ability to produce bile salt hydrolase activity were identified by 16S rRNA gene sequencing. The rRNA gene sequencing was determined using BLAST software compared to Eztexon-e database. Identification by 16S rDNA gene of selected lactic acid bacteria showed that MN and MN2 isolate were closely related to *Lactobacillus paraplantarum* DMS 10667$^{T}$ and *Lactobacillus plantarum* subsp. argentoratensis DK0 22$^{T}$ with similarity scores of 100% for both isolates. Isolate SM show 99.78% similarity scores to *Lactobacillus gasseri* ATCC 33323$^{T}$. Accordingly, 3 selected lactic acid bacterial isolates were identified and belonging to genera Lactobacillus as presented in table 8.

Table 8 Genotypic identification of LAB isolates on 16S rRNA gene sequencing

| Isolate no. | Closely species | Similarity |
|---|---|---|
| MN | *Lactobacillus paraplantarum* DSM 10667$^{T}$ | 100% |
| MN2 | *Lactobacillus plantarum* subsp. argentoratensis DK0 22$^{T}$ | 100% |
| SM | *Lactobacillus gasseri* ATCC 33323$^{T}$ | 99.78% |

**4. Phylogenetic analysis in molecular evolutionary genetics**

Phylogenetic tree analysis was performed to reveal the relationship between the representative isolates and known reference strain. The Phylogenetic tree analysis was constructed with neighbor-joining as inferred by the neighbor- joining method. Bootstrap values (expresses as percentages of 1,000 replication) 3 selected lactic acid bacterial exhibit cluster of Lactobacillus (Fig. 12)

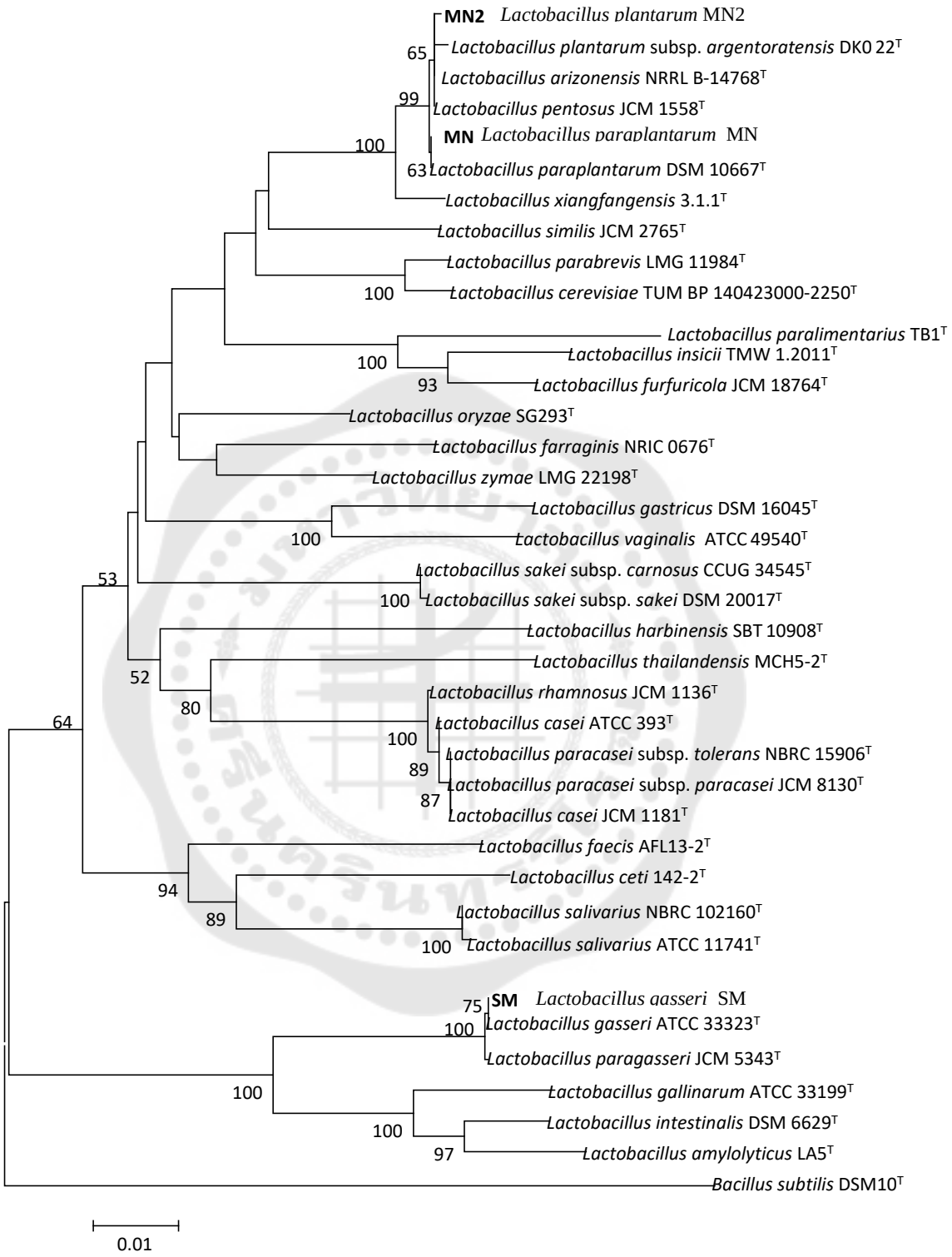



Figure 12 Phylogenetic tree based on 16S rRNA gene sequences.

Phylogenetic tree based on 16S rRNA gene sequences *showing* the relationships of the isolates strain MN, MN2 and SM with their closest relatives among the genus Lactobacillus. The phylogenetic tree was constructed using software MEGA 7.0 by the neighbour-joining method

**5. Acid and bile tolerance test**

The resistance to acid and bile in the human gastrointestinal tract constitutes key selection criteria for probiotic bacteria.

**5.1 Acid tolerance**

Acid tolerance of the 3 isolates lactic acid bacteria were investigated (table 9. and figures 13-15). In this study three tested isolates exhibited low pH tolerance. Isolates of lactic acid bacteria including, MN, MN2 and SM were incubated in MRS broth at pH 2.0, 3.0 and 4.0 for 3 h At pH 4.0 and pH 3.0 all isolates demonstrated significant growth reduction compared to those incubated in MRS condition. At pH 2.0, all isolates did not survive. In addition, at pH 3.0 isolate SM showed the highest tolerance among the three strains.

Table 9 Acid tolerance of selected lactic acid bacteria.

| Isolate no. | Resistance to acid | | | |
|---|---|---|---|---|
| | MRS control | pH 2.0 | pH 3.0 | pH 4.0 |
| MN | $8.32 \pm 0.05^{Aa}$ | $0^{Ba}$ | $8.13 \pm 0.00^{Ca}$ | $7.78 \pm 0.01^{Da}$ |
| MN2 | $8.31 \pm 0.05^{Aa}$ | $0^{Ba}$ | $8.06 \pm 0.03^{Ca}$ | $7.81 \pm 0.03^{Da}$ |
| SM | $8.31 \pm 0.01^{Aa}$ | $0^{Ba}$ | $8.13 \pm 0.00^{Ca}$ | $7.88 \pm 0.01^{Da}$ |

Each value in the table represents the mean value ± Standard Deviation (SD). Numbers (log10) of lactic acid bacteria. Mean values followed by difference superscript uppercase and lowercase letters in row for different acid concentration and columns for lactic acid bacteria, respectively, are significantly different ($p < 0.05$).

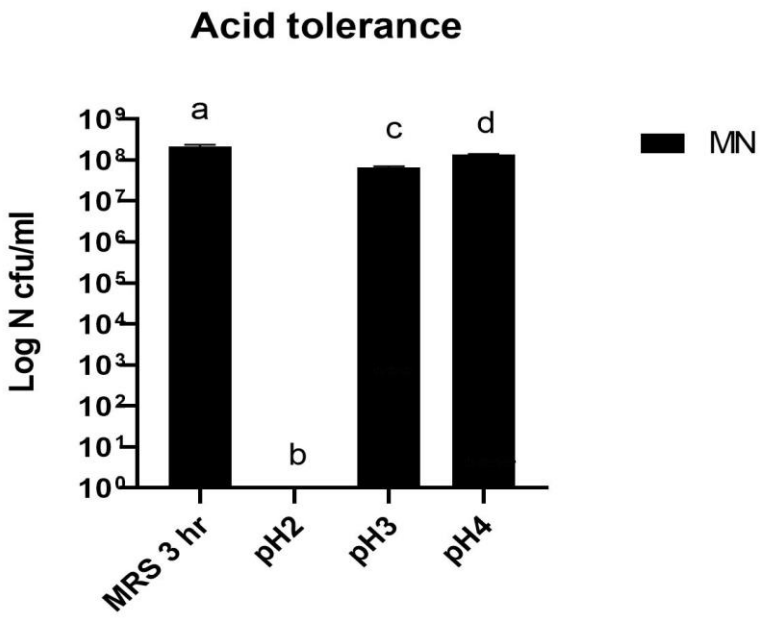



Figure 13 Acid tolerance of isolate MN. Values with a different letter are significantly different ($p < 0.05$).

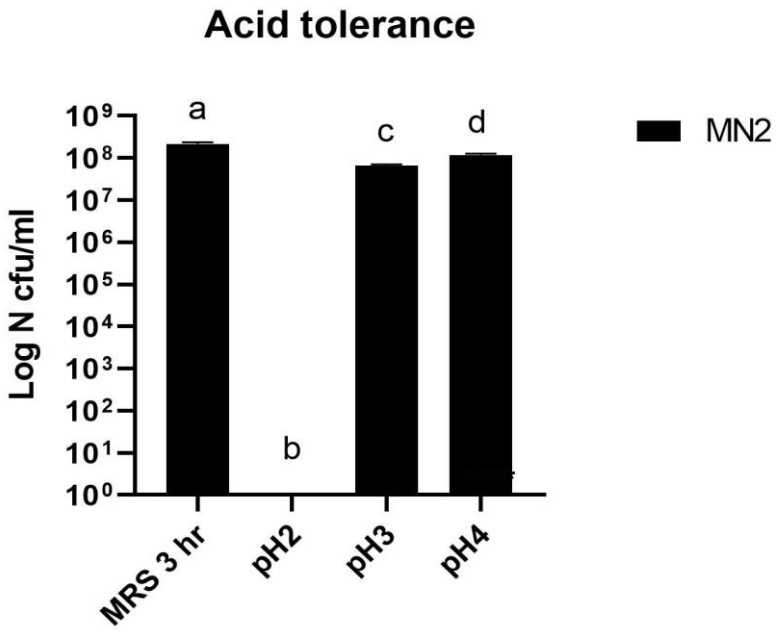



Figure 14 Acid tolerance of isolate MN2. Values with a different letter are significantly different ($p < 0.05$).

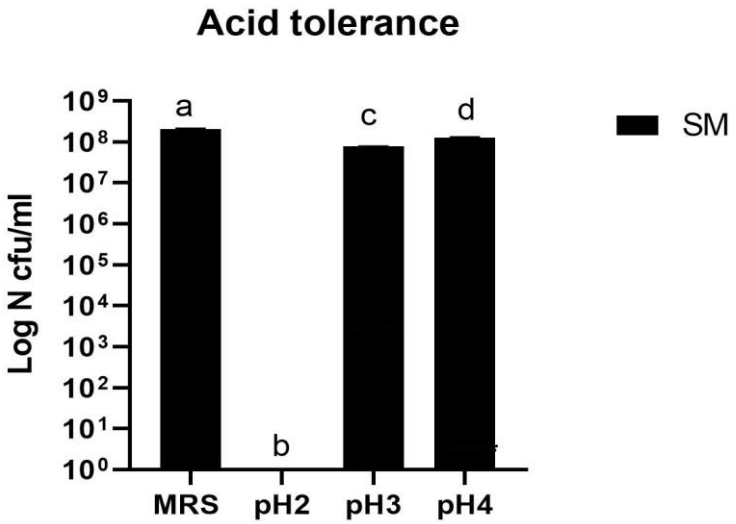



Figure 15 Acid tolerance of isolate SM. Values with a different letter are significantly different ($p < 0.05$).

**5.2 Bile tolerance**

Bile tolerance of the three isolates was also Bile tolerance of the three isolates was investigated, and the results are shown in table 10 and figures 16-18. The results showed that all isolates demonstrated significant reduced growth viable cells ($p<0.05$) from MRS when cultured with 0.3% and 0.8% bile concentration. The survival of all isolates decreased about 1 log values in 0.8 % of bile salt.

Table 10 Bile tolerance of selected lactic acid bacteria.

| Isolate no. | Resistance to bile | | |
|---|---|---|---|
| | MRS | 0.3% | 0.8% |
| MN | $8.36\pm0.03^{Aa}$ | $7.87\pm0.02^{Ba}$ | $7.02\pm0.02^{Ca}$ |
| MN2 | $8.38\pm0.01^{Aa}$ | $7.85\pm0.3^{Ba}$ | $6.99\pm0.06^{Ca}$ |
| SM | $8.34\pm0.05^{Aa}$ | $7.77\pm0.02^{Ba}$ | $6.87\pm0.04^{Ca}$ |

Each value in the table represents the mean value ± Standard Deviation (SD). Numbers (log10) of lactic acid bacteria. Mean values followed by difference superscript uppercase and lowercase letters in row for different ox gall concentration and columns for lactic acid bacteria, respectively, are significantly different ($p < 0.05$).

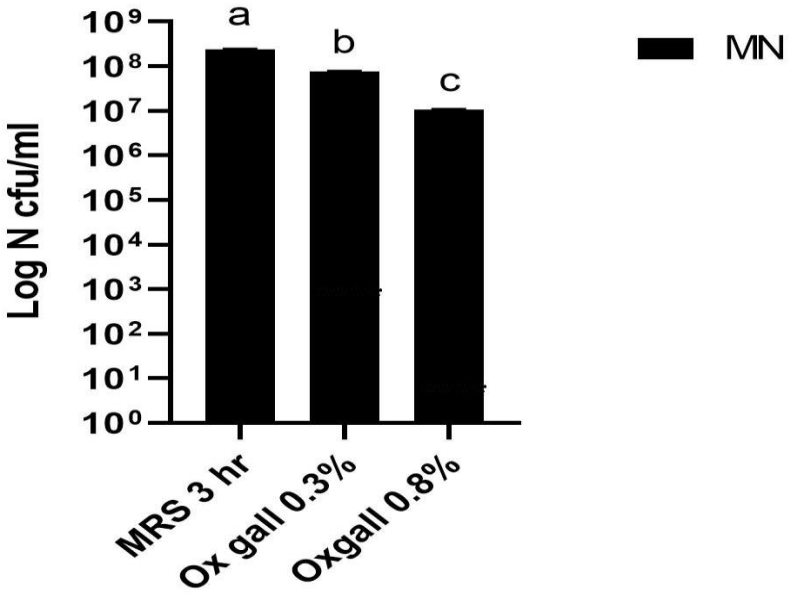



Figure 16 Bile tolerance of isolate MN. Values with a different letter are significantly different ($p < 0.05$).

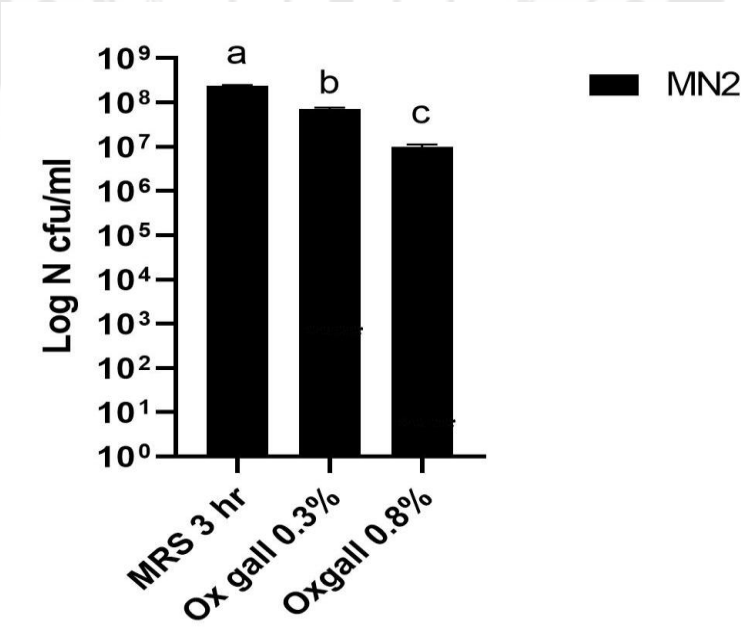



Figure 17 Bile tolerance of isolate MN2. Values with a different letter are significantly different ($p < 0.05$).

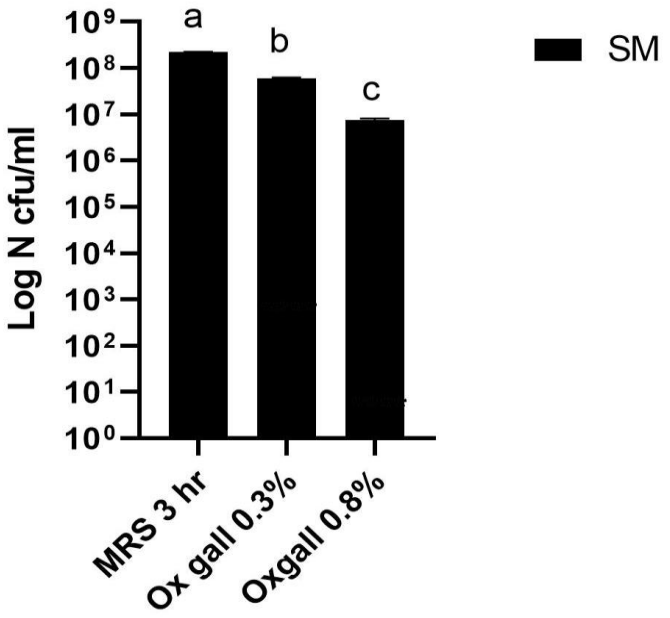



Figure 18 Bile tolerance of isolate SM. Values with a different letter are significantly different ($p < 0.05$).

**6. Cell surface hydrophobicity**

Microbial adhesion to biological surfaces were an important criterion for selection of probiotic strains. In the present study, isolates were evaluated for cell surface properties toward hydrocarbon xylene. The percent hydrophobic value of MN, MN2 and SM was 64.26%, 57.80% and 49.90% respectively. Isolate MN showed maximum affinity toward xylene (64.26%) than other isolates (Table 11).

Table 11 Cell surface hydrophobicity of isolates lactic acid bacteria.

| Isolate no. | Hydrophobicity (%) |
|---|---|
| MN | 64.26 |
| MN2 | 57.80 |
| SM | 49.90 |

**7. Antibiotic susceptibility**

Selected lactic acid bacteria showed resistance to nalidixic acid and streptomycin. Also, maximum susceptibility of all isolates was observed against chloramphenicol, tetracycline, ampicillin and gentamicin (Table 12.).

Table 12 Antibiotic susceptibility pattern of selected lactic acid bacteria

| Antibiotics | Concentration (μg/disc) | MN | MN2 | SM |
|---|---|---|---|---|
| Chloramphenicol | 30 | S | S | S |
| Tetracycline | 30 | S | S | S |
| Nalidixic acid | 30 | R | R | R |
| Ampicillin | 10 | S | S | S |
| Gentamicin | 10 | S | S | S |
| Streptomycin | 10 | R | R | R |

*R, resistant; S, sensitive*

**8. Hemolytic activity**

Potential probiotics should be safe and non-pathogenic. All the three isolates were incapable of exhibiting hemolysis on the agar media containing 5% blood. Therefore, selected LAB do not exhibit pathogenicity and are safe for consumption.

All selected lactic acid bacteria demonstrated non – hemolytic (γ-hemolysis) as confirmed by zone of hemolytic pattern (Fig.19-21).

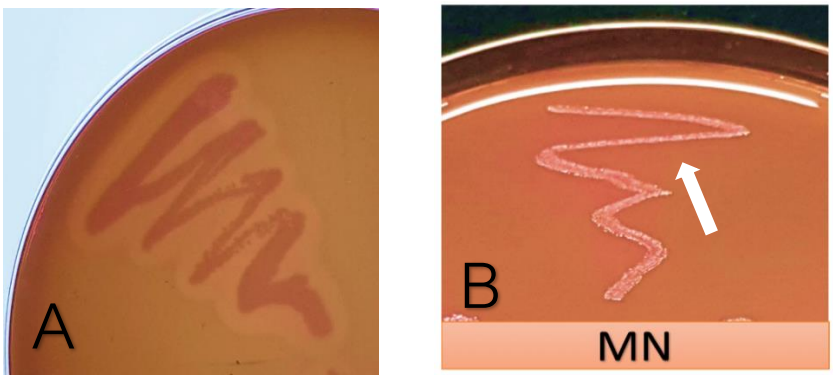



Figure 19 Hemolytic activities of isolate MN on Blood agar; no zone of clearing around the colony (γ-hemolysis) were presented by white arrow.

A: Hemolytic Control plate

B: Non-hemolytic plate

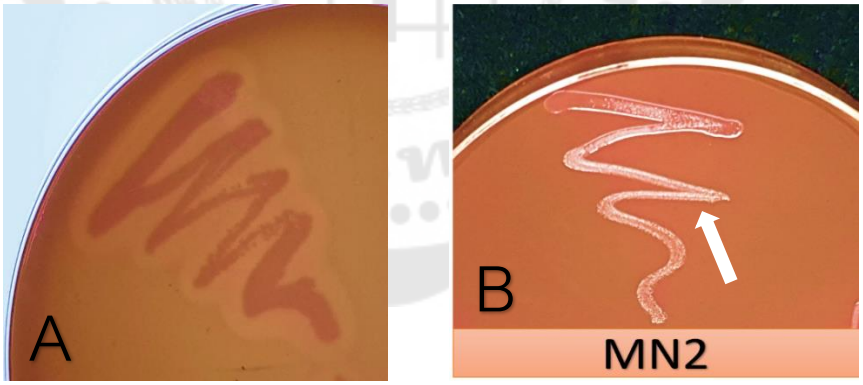



Figure 20 Hemolytic activities of isolate MN2 on Blood agar; no zone of clearing around the colony (γ-hemolysis) were presented by white arrow.

A: Plate hemolytic Control

B: Plate non-hemolytic

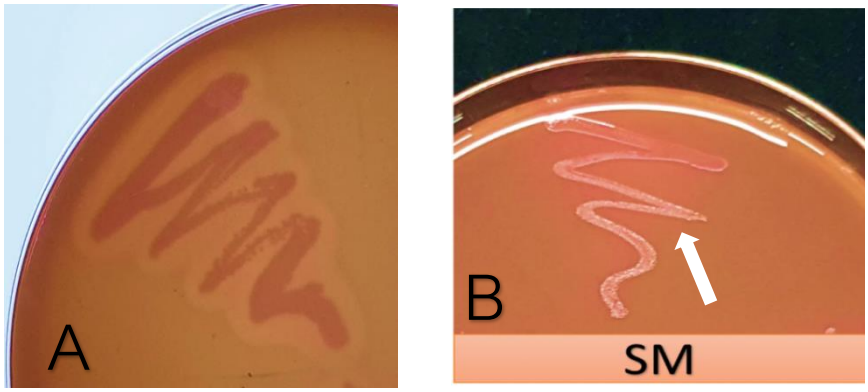



Figure 21 Hemolytic activities of isolate SM on Blood agar; no zone of clearing around the colony (γ-hemolysis) were presented by white arrow.

A: Plate hemolytic Control

B: Plate non-hemolytic

**9. Antimicrobial activity against pathogens**

Isolates were tested for antimicrobial activity against pathogens (table13.). Isolates MN2 presented non-antimicrobial activity against pathogens as observed from showed non-inhibit zone against all pathogens. However, isolate MN showed poor inhibition against *Shigella dysenteria* and isolate SM showed low inhibition against *Vibrio parahaemolyticus* and *Shigella dysenteria*.

Table 13 Antimicrobial activity against pathogens of isolated lactic acid bacteria and zone of inhibition (ZOI) against tested pathogens.

| Isolate no. | Tested bacterial strains (with ZOI in mm) | | |
|---|---|---|---|
| | MN | MN2 | SM |
| *Bacillus subtilis* ATCC 6633 | - | - | - |
| *Escherichia coli* ATCC 25922 | - | - | - |
| *Staphylococcus aureus* ATCC 25423 | - | - | - |
| *Vibrio Cholera* DMST 2873 | - | - | - |
| *Vibrio parahaemolyticus* DMST 5665 | - | - | + |

Table 13 (Continued)

| Isolate no. | Tested bacterial strains (with ZOI in mm) | | |
|---|---|---|---|
| | MN | MN2 | SM |
| *Proteus mirabilis* ATCC 13315 | - | - | - |
| *Pseudomonas aeruginosa* ATCC 21853 | - | - | - |
| *Helicobacter pylori* H40 | - | - | - |
| *Staphylococcus aureus* MRSA DSMT 20654 | - | - | - |
| *Streptococcus pyogenes* A 034 | - | - | - |
| *Shigella dysenteria* DMST 15111 | + | - | + |
| *salmonella typhi* DMST 5781 | - | - | - |

ZOI, Zone of inhibition, — no effect detected, +, diameter of inhibition zone 5 -10 mm; ++, 11-17 mm; +++, > 17 mm.

**10. Fermented milk products using probiotics**

Table. 14 presents fermentation times (to reach pH 4.6) for the prepared fermented milks (initial pH of milk was 6.74). Fermented milk with mixture culture used the shortest fermentation time. Long fermentation times (21 h) were observed in SM.

Table 14 Fermentation times (means ± SD) to reach pH 4.5 for selected probiotic lactic acid bacteria of single culture and mixed culture used in the manufacturing of fermented milks.

| Isolate no | Fermented times (h) |
|---|---|
| MN | 18.5 ±0.070 |
| MN2 | 19.5±0.070 |
| SM | 21±1.414 |
| Mixture culture | 16.25 ±0.353 |

**11. The quality parameters of probiotic lactic acid bacteria in fermented milk**

**11.1. Viability lactic acid bacteria in fermented milk products during storage**

Figure 22 presents changes in Lactic acid bacteria counts during fermentation and storage of fermented milk. Inoculation was initially at ~ log109 CFU/mL: counts 24 h after 24 h At day 1, LAB counts were at 9.28 9.33 9.43 and 9.42 log CFU/g for MN, MN2, SM and MIX, respectively (table 15). However, on 28 days of storage the fermented milk with SM has the highest number of bacteria counts.

Table 15 Viability of lactic acid bacteria in fermented milks during storage.

| Storage (day) | Viability of fermented milks product | | | |
|---|---|---|---|---|
| | MN | MN2 | SM | Mixed culture |
| 1 | $9.28 \pm 0.04^{Aa}$ | $9.33 \pm 0.08^{Aa}$ | $9.43 \pm 0.03^{Aa}$ | $9.42 \pm 0.03^{Aa}$ |
| 7 | $9.25 \pm 0.03^{Aa}$ | $9.25 \pm 0.06^{Aa}$ | $9.32 \pm 0.14^{Aab}$ | $9.28 \pm 0.01^{Aab}$ |
| 14 | $9.09 \pm 0.02^{Aa}$ | $9.12 \pm 0.06^{Aab}$ | $9.23 \pm 0.05^{Abc}$ | $9.16 \pm 0.02^{Ab}$ |
| 21 | $8.77 \pm 0.10^{Ab}$ | $8.94 \pm 0.13^{Ab}$ | $8.95 \pm 0.03^{Ac}$ | $9.07 \pm 0.05^{Ab}$ |
| 28 | $8 \pm 0^{Ac}$ | $8 \pm 0^{Ac}$ | $8.30 \pm 0.21^{Ad}$ | $8 \pm 0^{Ac}$ |

Each value in the table represents the mean value ± Standard Deviation (SD). Numbers (log10) of lactic acid bacteria in fermented milk. Mean values followed by difference superscript uppercase and lowercase letters in row for different fermented milk with lactic acid bacteria and columns for time (day), respectively, are significantly different ($p < 0.05$).

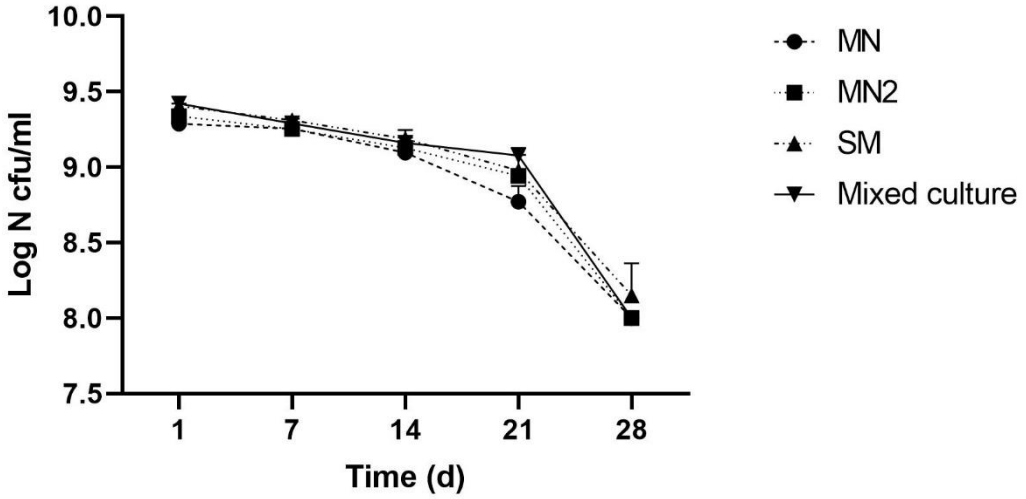



Figure 22 Viability of lactic acid bacteria in fermented milks during storage.
● MN: fermented milk with *Lactobacillus paraplantarum* ■ MN: fermented milk with *Lactobacillus plantarum* ▲ SM: fermented milk with *Lactobacillus gasserri* ▼ Mixed culture: fermented milk Mixed culture

**11.2 pH of fermented milk products during storage**

pH values of fermented milks decreased during storage. Significant differences ($P < 0.0001$) in the pH of fermented milk during storage were detected. Fermented milk with MN, MN2, SM and Mixture culture obtained pH decrease during 28 days of storage (5°C) when the initial pH values were 4.6. Over the total storage period, the most changes in pH were seen in fermented milk with MN, MN2, SM and Mixture culture, which it decreased from 4.6 in day 1 to 4, 4, 3.98 and 4.05 respectively in 28 days of during storage. Fermented milk that include isolate SM presented the highest acidity values in storage for 28 days. Table 16 and Figure 23 show the tendency of fermented milk pH to reduce during storage.

Table 16 pH in fermented milks during storage

| Storage (day) | pH fermented milk product | | | |
|---|---|---|---|---|
| | MN | MN2 | SM | Mixed culture |
| 1 | $4.6 \pm 0.021^{Aa}$ | $4.60 \pm 0.021^{Aa}$ | $4.61 \pm 0.041^{Aa}$ | $4.6 \pm 0.095^{Aa}$ |
| 7 | $4.4 \pm 0.057^{Ab}$ | $4.38 \pm 0.021^{Ab}$ | $4.33 \pm 0.013^{Ab}$ | $4.47 \pm 0.135^{Ab}$ |
| 14 | $4.3 \pm 0.021^{Ab}$ | $4.29 \pm 0.042^{Ab}$ | $4.24 \pm 0.036^{Ac}$ | $4.3 \pm 0.031^{Ac}$ |
| 21 | $4.1 \pm 0.024^{Ac}$ | $4.1 \pm 0.042^{Ac}$ | $4.1 \pm 0.088^{Ac}$ | $4.16 \pm 0.088^{Ad}$ |
| 28 | $4 \pm 0.028^{Ad}$ | $4 \pm 0.021^{Ac}$ | $3.98 \pm 0.21^{Ad}$ | $4.05 \pm 0.212^{Ae}$ |

Each value in the table represents the mean value ± Standard Deviation (SD). Numbers (log10) of lactic acid bacteria in fermented milk. Mean values followed by difference superscript uppercase and lowercase letters in row for different fermented milk with lactic acid bacteria and columns for time (day), respectively, are significantly different ($p < 0.05$).

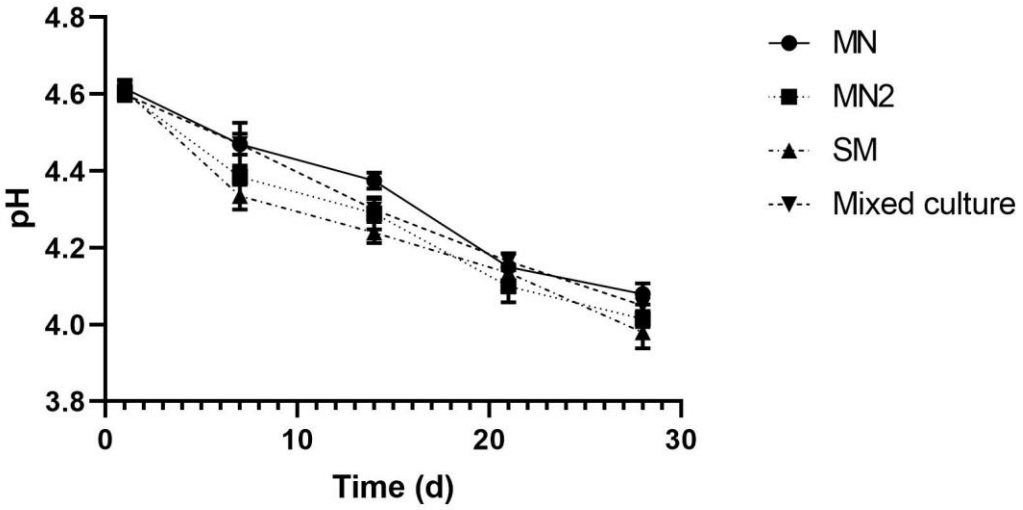



Figure 23 pH changes of fermented milks during storage.

● MN: fermented milk with *Lactobacillus paraplantarum* ■ MN: fermented milk with *Lactobacillus plantarum* ▲ SM: fermented milk with *Lactobacillus gasserri* ▼ Mixed culture: fermented milk Mixed culture

**11.3 Acid and bile tolerance of probiotic lactic acid bacteria in fermented milk.**

**11.3.1 Acid tolerance assay of selected LAB in fermented milk.**

The effect of acidic conditions on the viability of probiotics lactic acid bacteria in fermented milk was shown (Table17). At pH3 of 28-day storage of fermented milk with MN2, SM and Mixed culture showed significant decrease on 14 days of storage and fermented milk with MN significant decrease on 7 days of storage. On 1 to 28 day of storage fermented milk with MN MN2, SM and MIX decreased log 0.79, 0.85, 1.17 and 0.95 respectively

Fermented milk with MN, MN2 and SM isolates not survive at pH 2 on 14 21 and 28 day of storage (table 18.). Fermented milk with Mixed culture did not survive at pH 2 on 14, 21, and 28 days of storage. However, fermented milk with Mixed culture was tolerance acid pH2 lowest (table18).

Table 17 Acid tolerance pH 3 of probiotic lactic acid bacteria in fermented milk products.

| Storage (day) | Acid tolerance pH 3 (3h) | | | |
|---|---|---|---|---|
| | MN | MN2 | SM | Mixed culture |
| 1 | $7.02 \pm 0.02^{Aa}$ | $7.17 \pm 0.08^{Aa}$ | $7.17 \pm 0.04^{Aa}$ | $7.10 \pm 0.09^{Aa}$ |
| 7 | $6.92 \pm 0.03^{Aab}$ | $6.95 \pm 0.06^{Ab}$ | $7.02 \pm 0.02^{Aa}$ | $7.05 \pm 0.13^{Aa}$ |
| 14 | $6.87 \pm 0.04^{Aab}$ | $6.81 \pm 0.04^{Abc}$ | $6.65 \pm 0.06^{Ab}$ | $6.92 \pm 0.03^{Aab}$ |
| 21 | $6.53 \pm 0.08A^{bc}$ | $6.65 \pm 0.06^{Abc}$ | $6.38 \pm 0.12^{Ab}$ | $6.53 \pm 0.08^{Abc}$ |
| 28 | $6.23 \pm 4.24^{Ac}$ | $6.32 \pm 4.45^{Ac}$ | $6. \pm 0^{Ab}$ | $6.15 \pm 0.21^{Ac}$ |

Each value in the table represents the mean value ± Standard Deviation (SD). Numbers (log10) of lactic acid bacteria in fermented milk. Mean values followed by difference superscript uppercase and lowercase letters in row for different fermented milk with lactic acid bacteria and columns for time (day), respectively, are significantly different ($p < 0.05$).

Table 18 Acid tolerance pH 2 of probiotic lactic acid bacteria in fermented milk products.

| Storage (day) | Acid tolerance pH 2 (3h) | | | |
|---|---|---|---|---|
| | MN | MN2 | SM | Mixed culture |
| 1 | $6.38 \pm 0.124^{Aa}$ | $6.15 \pm 0.212^{Aa}$ | $6.38 \pm 0.124^{Aa}$ | $6.38 \pm 0.124^{Aa}$ |
| 7 | $6.15 \pm 0.212^{Aa}$ | $6.1 \pm 0.212^{Aa}$ | $6.23 \pm 0.088^{Aa}$ | $6^{Ab}$ |
| 14 | $0^{Ab}$ | $0^{Ab}$ | $0^{Ab}$ | $0^{Ac}$ |
| 21 | $0^{Ab}$ | $0^{Ab}$ | $0^{Ab}$ | $0^{Ac}$ |
| 28 | $0^{Ab}$ | $0^{Ab}$ | $0^{Ab}$ | $0^{Ac}$ |

Each value in the table represents the mean value ± Standard Deviation (SD). Numbers (log10) of lactic acid bacteria in fermented milk. Mean values followed by difference superscript uppercase and lowercase letters in row for different fermented milk with lactic acid bacteria and columns for time (day), respectively, are significantly different ($p < 0.05$).

### 11.3.2 Bile tolerance assay of selected LAB in fermented milk.

Bile tolerance 0.3 % and 0.8% of fermented milks with probiotic lactic acid bacteria 3 h exposure was demonstrated in table 19 and 20 present. Twenty - eight day of storage fermented milk with MN, MN2, SM and Mixed culture showed cells number decreased when exposure to ox gall 0.3% and 0.8%. At ox gall 0.3% the fermented milk of all isolates showed significant decrease on 7 days of storage. At ox gall 0.3% on 1 to 28 day of storage fermented milk with MN, MN2, SM and MIX decreased log 1.22, 0.92, 1.39 and 1.42 respectively (table19.).

At ox gall 0.8 % fermented milk with MN SM and Mixed culture showed significant decrease on 7 days of storage and fermented milk with MN2 significant decrease on 14 days of storage. At ox gall 0.8% on 1 to 28 day of storage fermented milk with MN, MN2, SM and Mixed culture decreased log 1.22 1.08 1.31 and 1.42 respectively. However, at ox gall 0.8% fermented milks with Mixed culture found survive cell lowest (table 20.).

Table 19 Bile Tolerance Ox gall 0.3 % test in fermented milk products during storage.

| Storage (day) | Bile tolerance of 0.3 % Ox gall (3h) | | | |
|---|---|---|---|---|
| | MN | MN2 | SM | Mixed culture |
| 1 | $8.60 \pm 0.30^{Aa}$ | $8.57 \pm 4.04^{Aa}$ | $8.54 \pm 0.06^{Aa}$ | $8.57 \pm 0.03^{Aa}$ |
| 7 | $8.47 \pm 0.04^{Ab}$ | $8.40 \pm 0.012^{Ab}$ | $8.45 \pm 0.03^{Ab}$ | $8.45 \pm 0.03^{Ab}$ |
| 14 | $8.30 \pm 0.03^{Ac}$ | $8.26 \pm 0.016^{Ab}$ | $8.19 \pm 0.01^{Abc}$ | $8.19 \pm 0.01^{Ac}$ |
| 21 | $7.81 \pm 0.04^{Ad}$ | $7.95 \pm 0.06^{Ac}$ | $7.99 \pm 0.06^{Ac}$ | $7.99 \pm 0.06^{Ac}$ |
| 28 | $7.38 \pm 0.12^{Ad}$ | $7.65 \pm 0.06^{Ac}$ | $7.15 \pm 0.88^{Ac}$ | $7.15 \pm 0.21^{Ad}$ |

Each value in the table represents the mean value ± Standard Deviation (SD). Numbers (log10) of lactic acid bacteria in fermented milk. Mean values followed by difference superscript uppercase and lowercase letters in row for different fermented milk with lactic acid bacteria and columns for time (day), respectively, are significantly different ($p < 0.05$).

Table 20 Bile Tolerance Ox gall 0.8 % test in fermented milk products during storage.

| Storage (day) | Bile tolerance of 0.8 % Ox gall (3h) | | | |
|---|---|---|---|---|
| | MN | MN2 | SM | Mixed culture |
| 1 | $8.37 \pm 0.025^{Aa}$ | $8.28 \pm 4.015^{Aa}$ | $8.31 \pm 0.014^{Aa}$ | $8.28 \pm 0.015^{Aa}$ |
| 7 | $8.26 \pm 0.016^{Ab}$ | $8.22 \pm 0.036^{Aa}$ | $8.16 \pm 0.021^{ABb}$ | $8.05 \pm 0.080^{Bb}$ |
| 14 | $7.97 \pm 0.032^{Ac}$ | $8.03 \pm 0.112^{Ab}$ | $8.03 \pm 0.055^{Ab}$ | $7.89 \pm 0.077^{Ab}$ |
| 21 | $7.65 \pm 0.068^{Ac}$ | $7.92 \pm 0.109^{Ab}$ | $7.92 \pm 0.036^{Ab}$ | $7.73 \pm 0.055^{Ab}$ |
| 28 | $7.15 \pm 0.212^{Ac}$ | $7.15 \pm 0.212^{Ac}$ | $7 \pm 0^{Ac}$ | $6.84 \pm 0.212^{Ac}$ |

Each value in the table represents the mean value ± Standard Deviation (SD). Numbers (log10) of lactic acid bacteria in fermented milk. Mean values followed by difference superscript uppercase and lowercase letters in row for different fermented milk with lactic acid bacteria and columns for time (day), respectively, are significantly different ($p < 0.05$).

**11.4 The existence of BSH activity probiotic lactic acid bacteria in fermented milk during storage.**

The existence of BSH activity in fermented milk was demonstrated in table 21. Fermented milk with all isolates showed existence of maximum BSH activity in fermented milk on 1, 7, 14, 21 days of storage and dwindle on 28 days of storage. However, on 28 day of storage fermented milk with MIX presented the least existence of BSH activity in fermented milk.

Table 21 The existence of BSH activity probiotic lactic acid bacteria in fermented milk during storage.

| Storage (day) | Bile salt hydrolase activity | | | |
|---|---|---|---|---|
| | MN | MN2 | SM | Mixed culture |
| 1 | +++ | +++ | +++ | +++ |
| 7 | +++ | +++ | +++ | +++ |
| 14 | +++ | +++ | +++ | +++ |
| 21 | +++ | +++ | +++ | +++ |
| 28 | ++ | ++ | ++ | + |

+++, Strong bile salt hydrolase activity; ++, modulate bile salt hydrolase activity; +, weak bile salt hydrolase activity

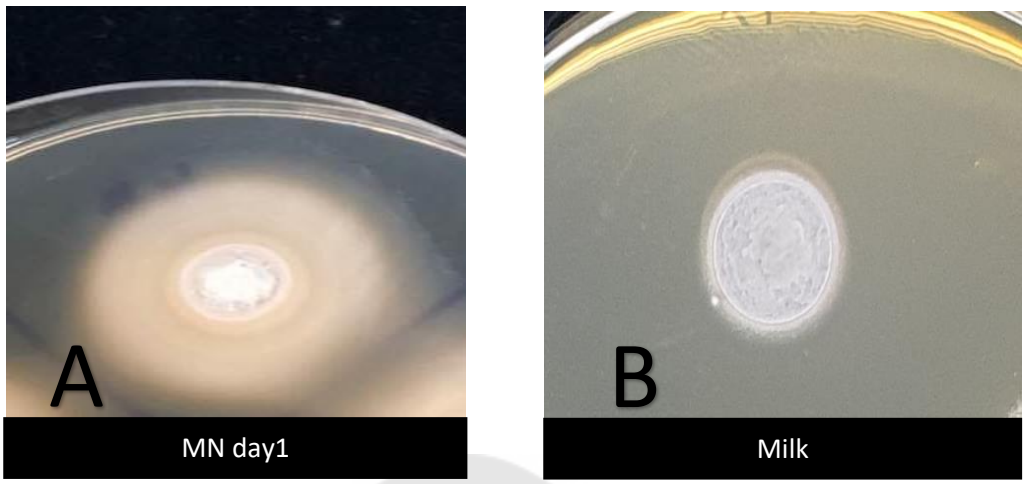



Figure 24 Bile salt hydrolase activity of fermented milks with MN (Storage day1) on MRS agar precipitation zone

A: plate containing 0.5% TDCA

B: Control

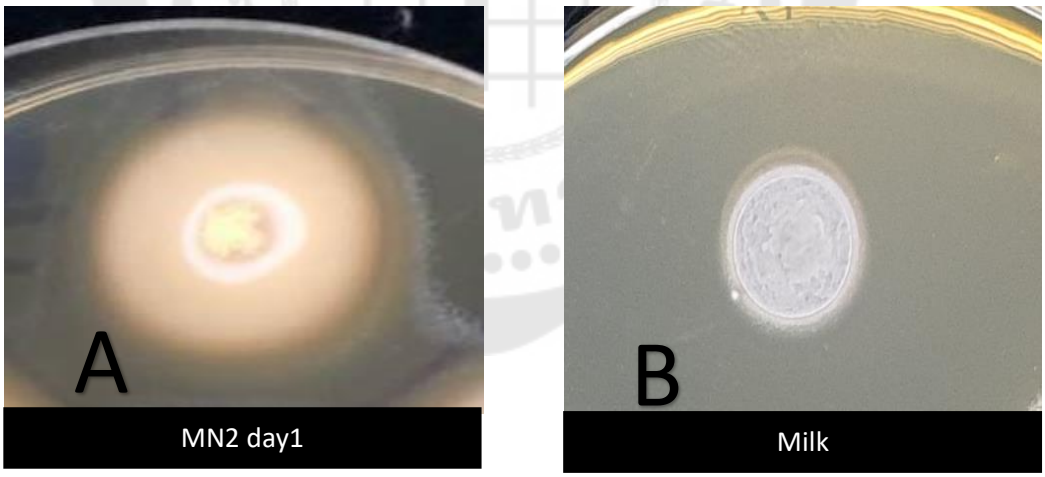



Figure 25 Bile salt hydrolase activity of fermented milks with MN2 (Storage day1) on MRS agar precipitation zone

A: plate containing 0.5% TDCA

B: Control

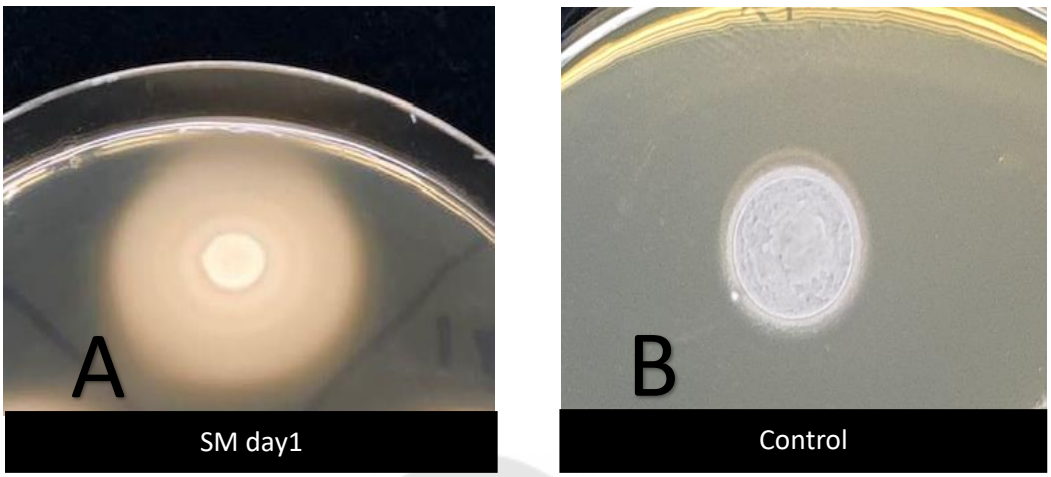



Figure 26 Bile salt hydrolase activity of fermented milks with SM (Storage day1) on MRS agar precipitation zone

A: plate containing 0.5% TDC

B: Control

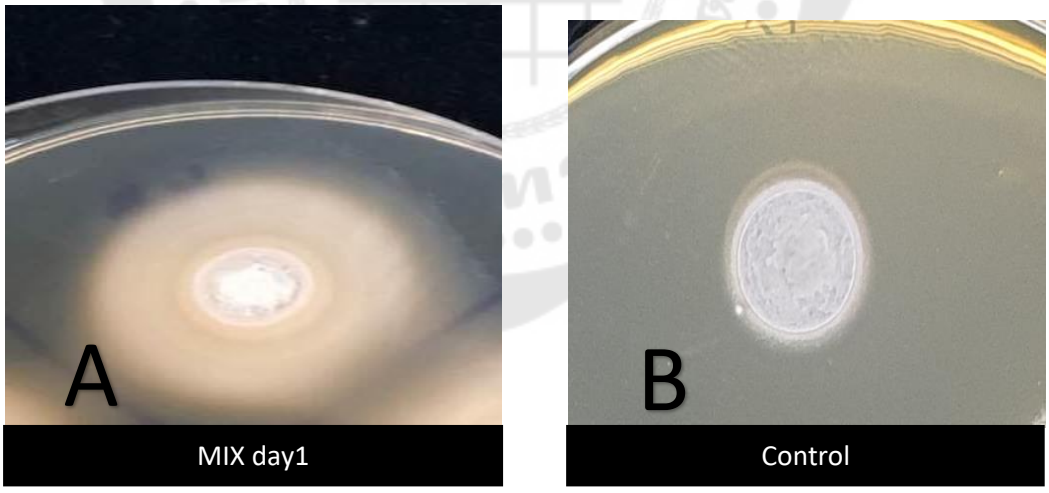



Figure 27 Bile salt hydrolase activity of fermented milks with Mixed culture (Storage day1) on MRS agar precipitation zone

A: plate containing 0.5% TDCA

B: Control

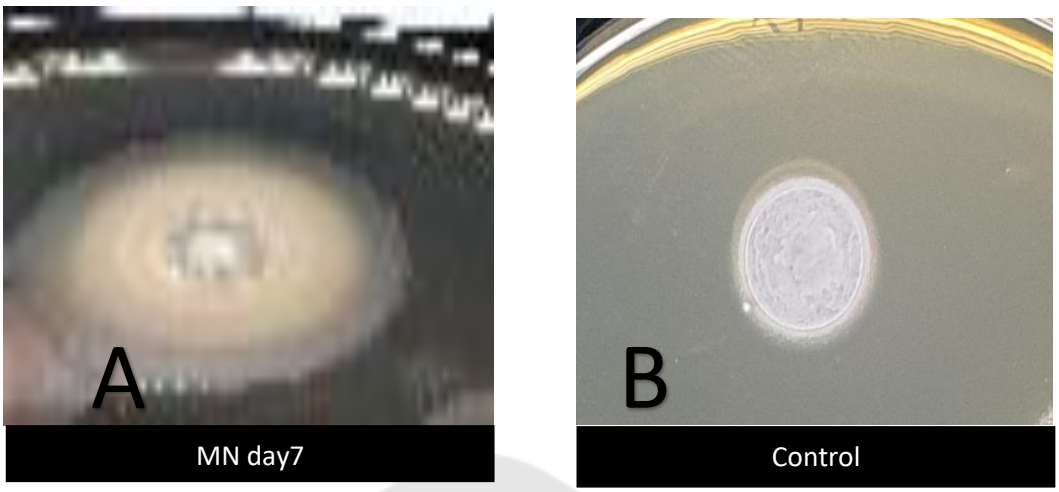



Figure 28 Bile salt hydrolase activity of fermented milks with MN (Storage day7) on MRS agar precipitation zone

A: plate containing 0.5% TDCA

B: Control

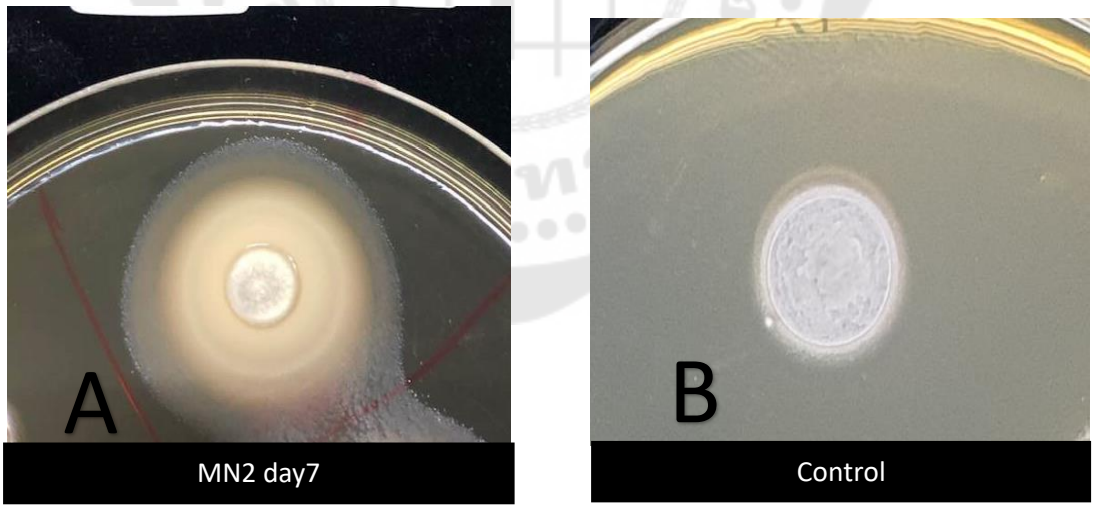



Figure 29 Bile salt hydrolase activity of fermented milks with MN2 (Storage day7) on MRS agar precipitation zone

A: plate containing 0.5% TDCA

B: Control

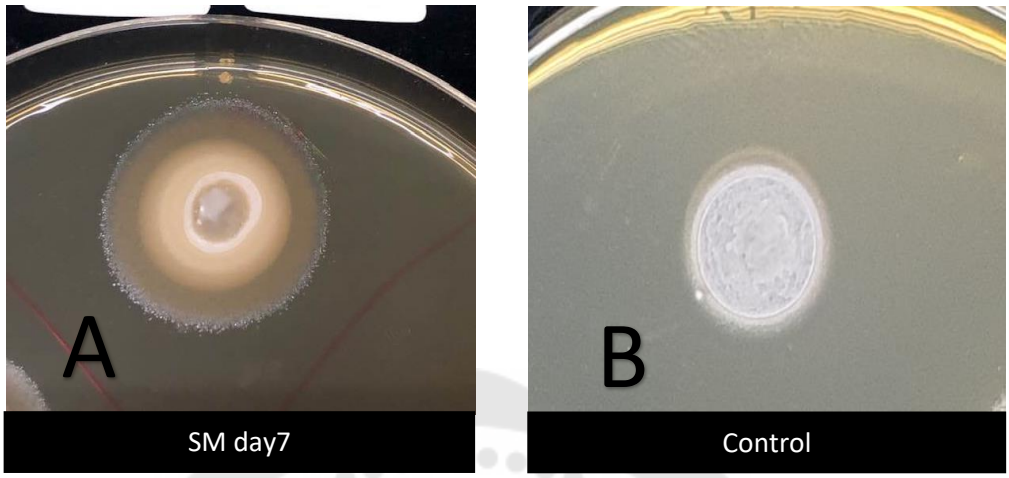



Figure 30 Bile salt hydrolase activity of fermented milks with SM (Storage day7) on MRS agar precipitation zone

A: plate containing 0.5% TDCA

B: Control

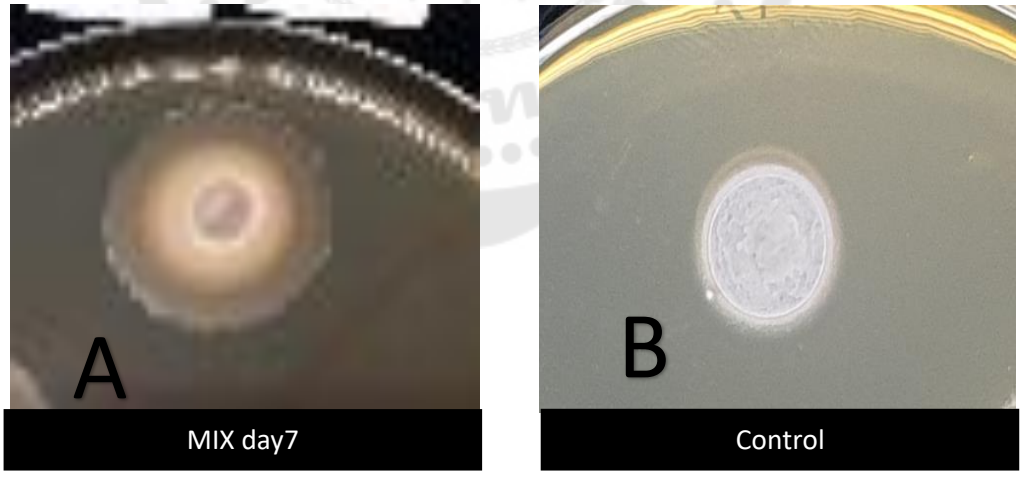



Figure 31 Bile salt hydrolase activity of fermented milks with Mixed culture (Storage day7) on MRS agar precipitation zone

A: plate containing 0.5% TDCA

B: Control

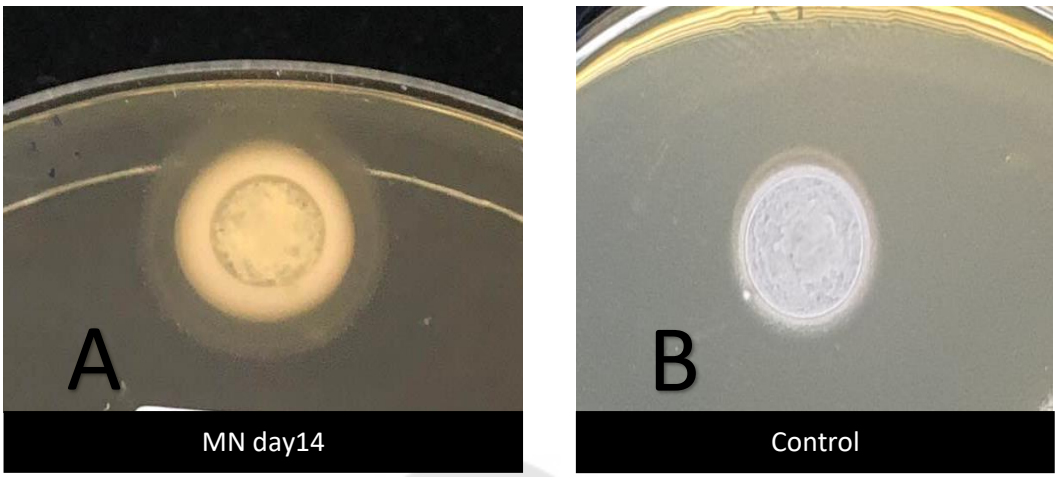



Figure 32 Bile salt hydrolase activity of fermented milks with MN (Storage day14) on MRS agar precipitation zone

A: plate containing 0.5% TDCA

B: Control

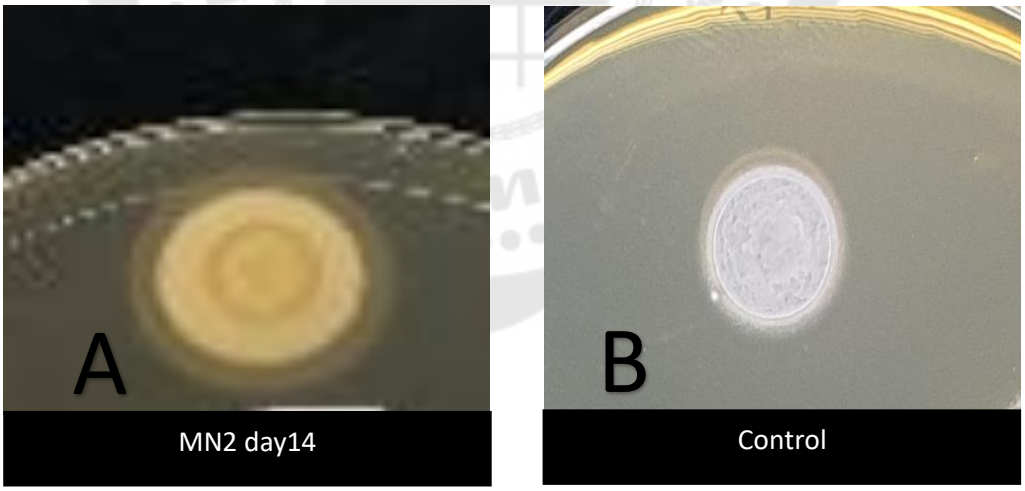



Figure 33 Bile salt hydrolase activity of fermented milks with MN2 (Storage day14) on MRS agar precipitation zone

A: plate containing 0.5% TDCA

B: Control

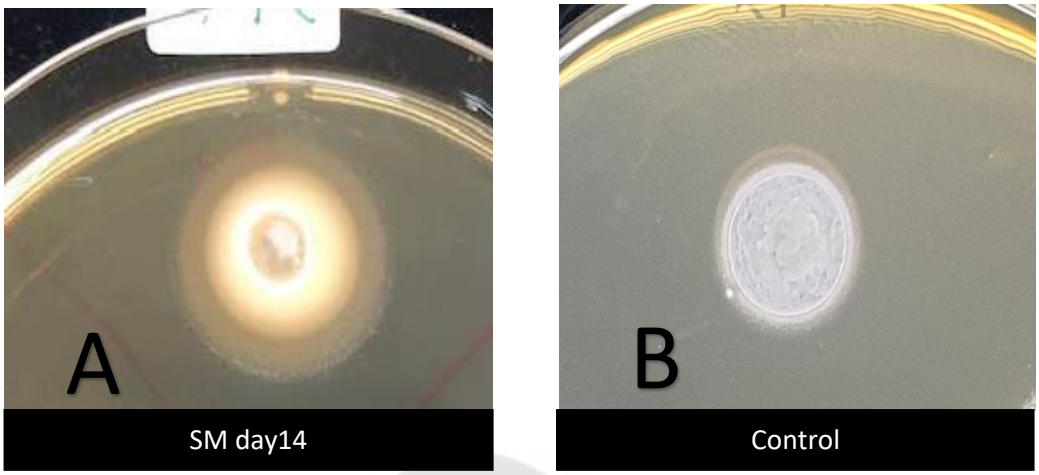



Figure 34 Bile salt hydrolase activity of fermented milks with SM (Storage day14) on MRS agar precipitation zone

A: plate containing 0.5% TDCA

B: Control

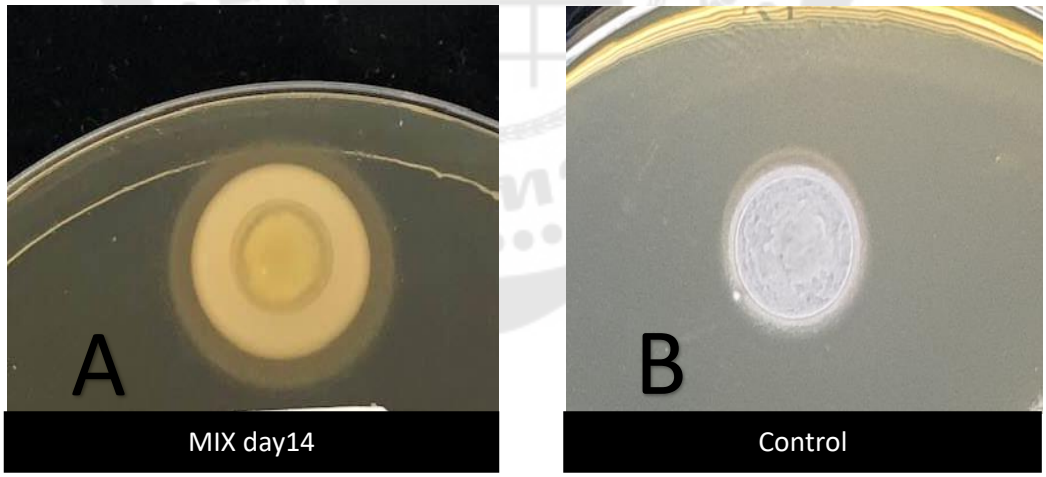



Figure 35 Bile salt hydrolase activity of fermented milks with Mixed culture (Storage day14) on MRS agar precipitation zone

A: plate containing 0.5% TDCA

B: Control

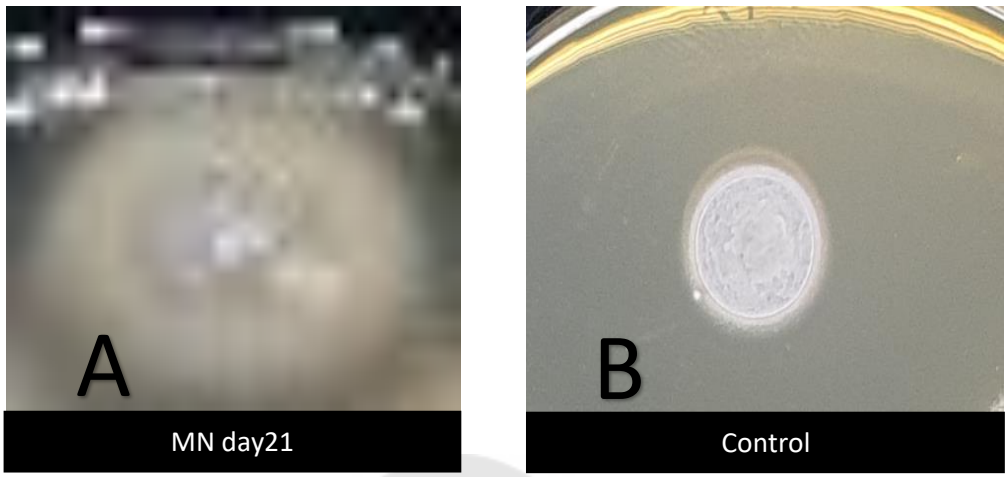



Figure 36 Bile salt hydrolase activity of fermented milks with MN (Storage day21) on MRS agar precipitation zone

A: plate containing 0.5% TDCA

B: Control

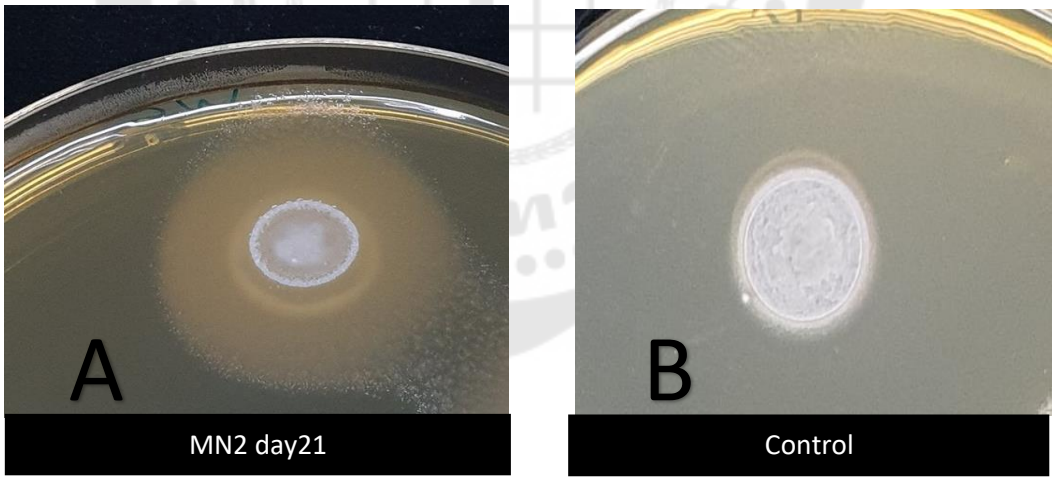



Figure 37 Bile salt hydrolase activity of fermented milks with MN2 (Storage day21) on MRS agar precipitation zone

A: plate containing 0.5% TDCA

B: Control

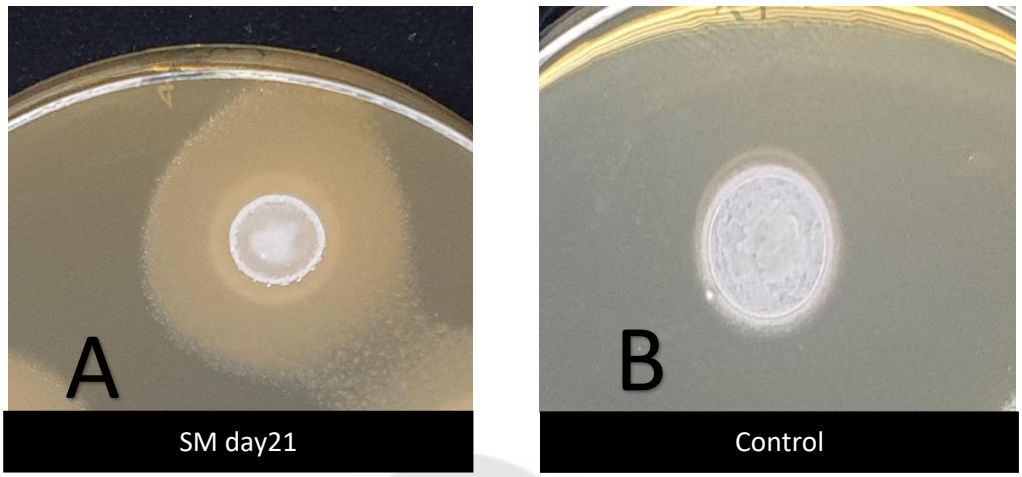



Figure 38 Bile salt hydrolase activity of fermented milks with SM (Storage day21) on MRS agar precipitation zone

A: plate containing 0.5% TDCA

B: Control

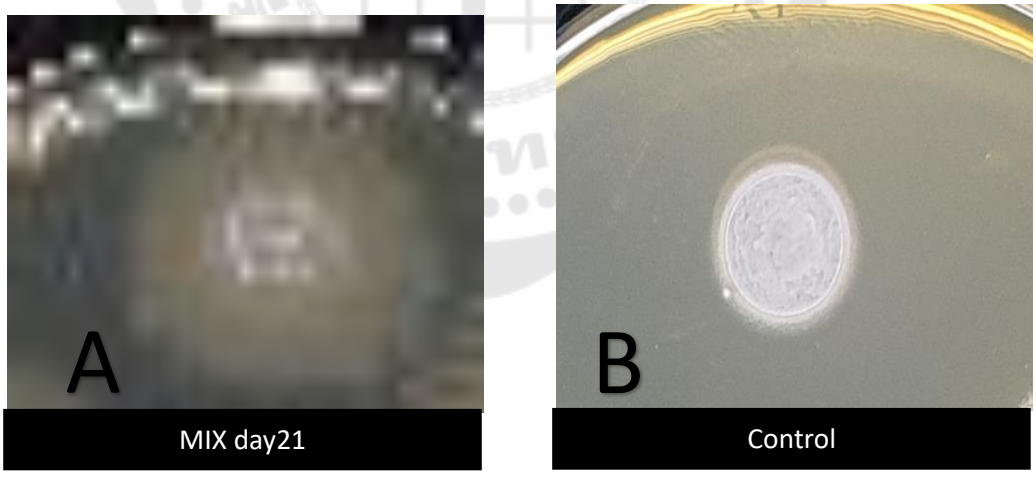



Figure 39 Bile salt hydrolase activity of fermented milks with Mixed culture (Storage day21) on MRS agar precipitation zone

A: plate containing 0.5% TDCA

B: Control

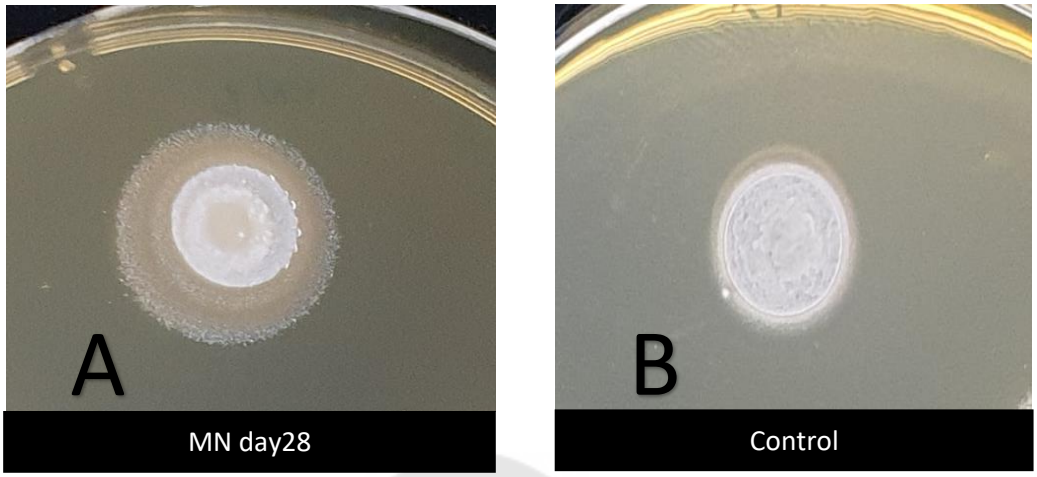



Figure 40 Bile salt hydrolase activity of fermented milks with MN (Storage day28) on MRS agar precipitation zone

A: plate containing 0.5% TDCA

B: Control

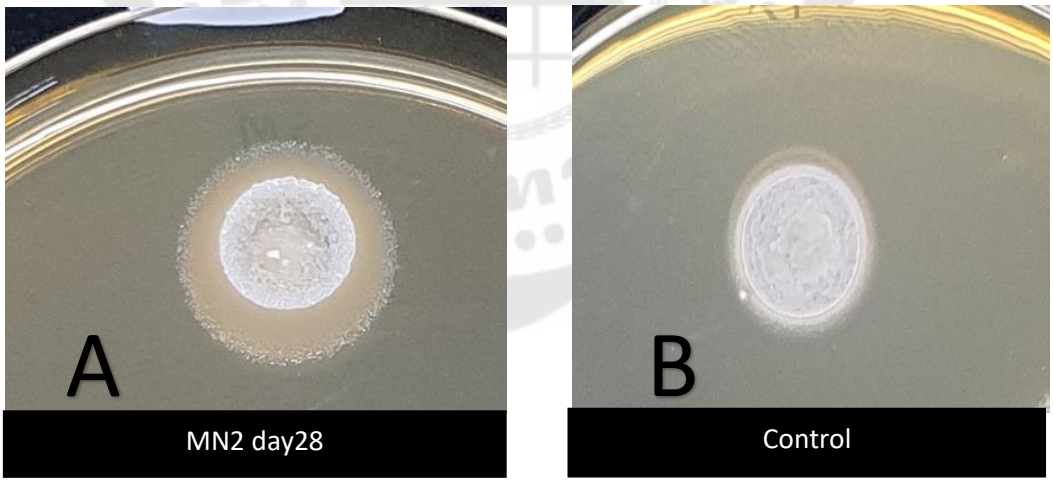



Figure 41 Bile salt hydrolase activity of fermented milks with MN2 (Storage day28) on MRS agar precipitation zone

A: plate containing 0.5% TDCA

B: Control

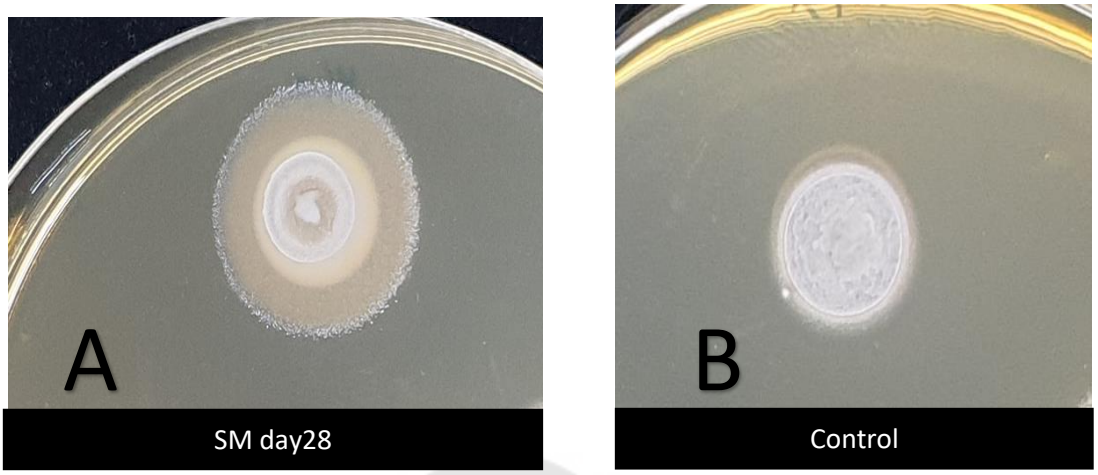



Figure 42 Bile salt hydrolase activity of fermented milks with SM (Storage day28) on MRS agar precipitation zone

A: plate containing 0.5% TDCA

B: Control

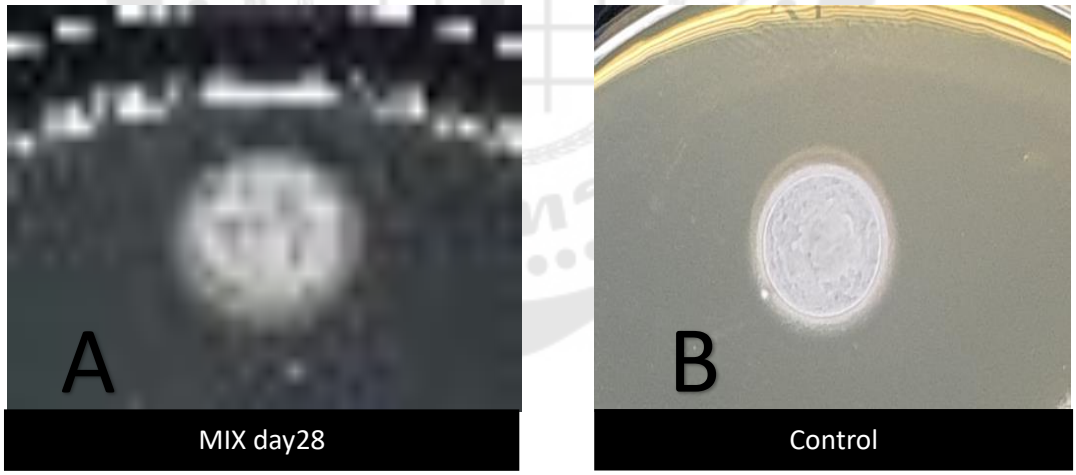



Figure 43 Bile salt hydrolase activity of fermented milks with Mixed culture (Storage day28) on MRS agar precipitation zone

A: plate containing 0.5% TDCA

B: Control

**11.5 Texture of fermented milk products.**

Texture is a very important characteristic of fermented milk. In the current study, the firmness values of fermented milk during 28 days storage are presented in table 22. Firmness of fermented with MN MN2 and MIX significantly increased ($p<0.05$) during 7-day storage and firmness of fermented with SM significantly increased ($p<0.05$) during 14-day storage. Fermented milk with mixed culture exhibited the highest values of firmness.

Table 22 Firmness of fermented milk during storage.

| Storage (day) | Firmness (g) | | | |
|---|---|---|---|---|
| | MN | MN2 | SM | Mixed culture |
| 1 | $40.75 \pm 0.91^{Aa}$ | $48.55 \pm 1.06^{Ba}$ | $49.35 \pm 1.90^{Ba}$ | $64.49 \pm 0.28^{Ca}$ |
| 7 | $47.30 \pm 2.68^{Ab}$ | $50.55 \pm 0\ 80^{Bab}$ | $51.4 \pm 0.42^{Ba}$ | $73.9 \pm 2.54^{Cb}$ |
| 14 | $46.05 \pm 0.49^{Ab}$ | $53.05 \pm 2.33^{Bb}$ | $62.20 \pm 1.27^{Cb}$ | $74.4 \pm 3.25^{Db}$ |
| 21 | $46.85 \pm 1.76^{Ab}$ | $54.20 \pm 0.70^{Bab}$ | $63.75 \pm 0.91^{Cb}$ | $76.3 \pm 0.28^{Db}$ |
| 28 | $49.20 \pm 0.14^{Ab}$ | $57.05 \pm 0.63^{Aab}$ | $63.65 \pm 0.35^{Bb}$ | $77.6 \pm 1.06^{Cb}$ |

Each value in the table represents the mean value ± Standard Deviation (SD). Mean values followed by difference superscript uppercase and lowercase letters in row for different fermented milk with lactic acid bacteria and columns for time (day), respectively, are significantly different ($p < 0.05$).

**11.6 Syneresis of fermented milk products**

Syneresis of fermented milks during storage was shown in table 23 and figure 44. Fermented milks with MN and mixed culture exhibited significant increases in % syneresis on day 7. Fermented milks with MN2 and SM significant increases in % syneresis on day 14.

Table 23 % Syneresis of fermented milks during storage.

| Storage (day) | %Syneresis | | | |
|---|---|---|---|---|
| | MN | MN2 | SM | Mixed culture |
| 1 | $0^{Aa}$ | $0^{Aa}$ | $0^{Aa}$ | $0^{Aa}$ |
| 7 | $3.3 \pm 0A^{Bab}$ | $5.5 \pm 1.92^{Ab}$ | $3.66 \pm 0.57^{ABb}$ | $0^{Ba}$ |
| 14 | $3.8 \pm 0.962^{Ab}$ | $6.66 \pm 0^{Ab}$ | $6.66 \pm 0^{Abc}$ | $5 \pm 0^{Ab}$ |
| 21 | $7.77 \pm 1.92^{Ac}$ | $10 \pm 3.3^{Ac}$ | $10 \pm 0^{Acd}$ | $6.6 \pm 0^{Abc}$ |
| 28 | $11.11 \pm 1.92^{Ac}$ | $12.22 \pm 1.92^{Ac}$ | $13 \pm 0^{Ad}$ | $10 \pm 0^{Ac}$ |

Each value in the table represents the mean value ± Standard Deviation (SD). Mean values followed by difference superscript uppercase and lowercase letters in row for different fermented milk with lactic acid bacteria and columns for time (day), respectively, are significantly different ($p < 0.05$).

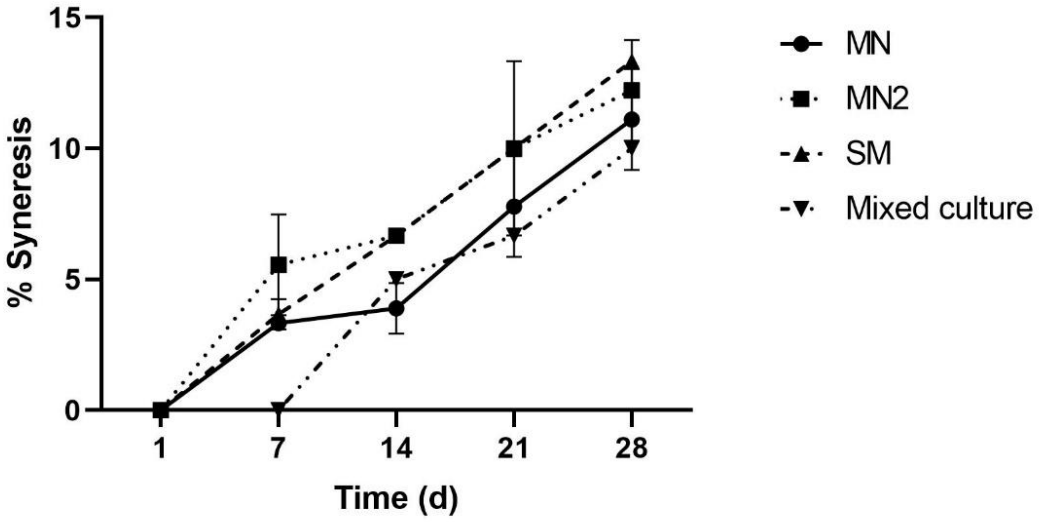



Figure 44 % Syneresis of fermented milks during storage

● MN: fermented milk with *Lactobacillus paraplantarum* ■ MN: fermented milk with *Lactobacillus plantarum* ▲ SM: fermented milk with *Lactobacillus gasserri* ▼ Mixed culture: fermented milk with mixed cultur

**11.7 Rheological properties of fermented milk products**

The elastic modulus (G′) of the fermented milk is presented in table G′ values significantly increased at higher frequencies of fermented milk with MN MN2 SM and MIX. G′ data indicated that all the samples showed a very slight dependency on frequency interval of 0.1–10 Hz (Fig.). The storage modulus (G′) of all samples increased

Table 24 Rheological characteristics of fermented milk product

| Storage (day) | Rheological characteristics (G' (Pa)) | | | |
|---|---|---|---|---|
| | MN | MN2 | SM | Mixed culture |
| 1 | $39.55^{Aa}$ | $54.36^{Aa}$ | $95.68^{Ba}$ | $77.36^{ABa}$ |
| 7 | $46.93^{Aa}$ | $64.20^{ABa}$ | $89.08^{Ba}$ | $101.47^{Bab}$ |
| 14 | $63.33A^{ab}$ | $67.71^{Aa}$ | $77.94^{Aa}$ | $99.75^{Aab}$ |
| 21 | $95.96^{Ab}$ | $73.26^{Aab}$ | $81.32^{Aa}$ | $102.08^{Aab}$ |
| 28 | $97.48^{Ab}$ | $109.14^{Ab}$ | $114.008^{Aa}$ | $133.35^{Ab}$ |

Difference superscript uppercase and lowercase letters in row for different fermented milk with lactic acid bacteria and columns for time (day), respectively, are significantly different ($p < 0.05$).

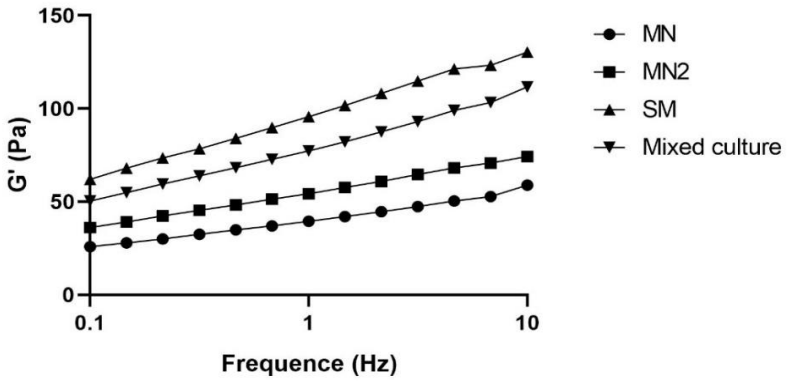



Figure 45 Dependence of G′ on frequency for fermented milk during storage on 1 day ● MN: fermented milk with *Lactobacillus paraplantarum* ■ MN: fermented milk with *Lactobacillus plantarum* ▲ SM: fermented milk with *Lactobacillus gasserri* ▼ Mixed culture: fermented milk with mixed culture

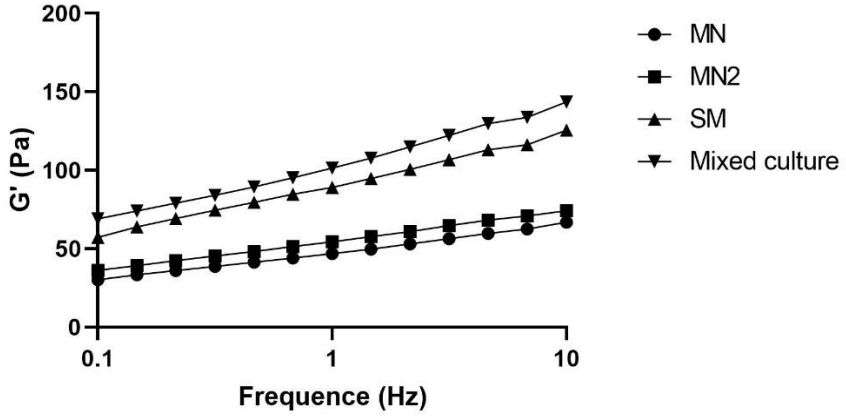



Figure 46 Dependence of G′ on frequency for fermented milk during storage on 7 days

● MN: fermented milk with *Lactobacillus paraplantarum* ■ MN: fermented milk with *Lactobacillus plantarum* ▲ SM: fermented milk with *Lactobacillus gasserri* ▼ Mixed culture: fermented milk with mixed culture

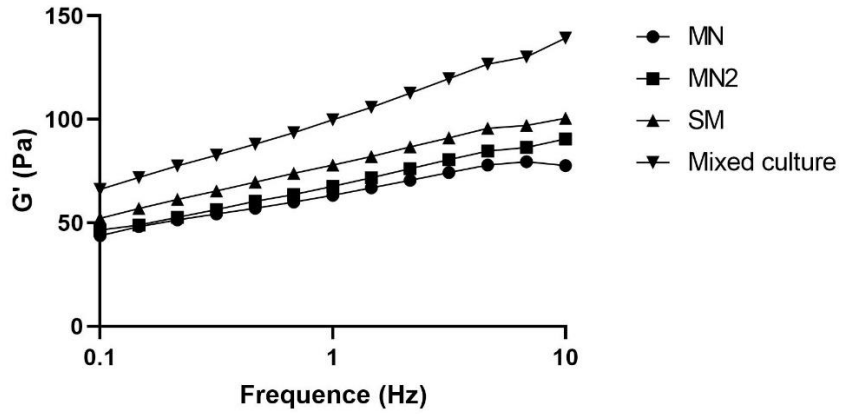



Figure 47 Dependence of G′ on frequency for fermented milk during storage on 14 days

● MN: fermented milk with *Lactobacillus paraplantarum* ■ MN: fermented milk with *Lactobacillus plantarum* ▲ SM: fermented milk with *Lactobacillus gasserri* ▼ Mixed culture: fermented milk with mixed culture

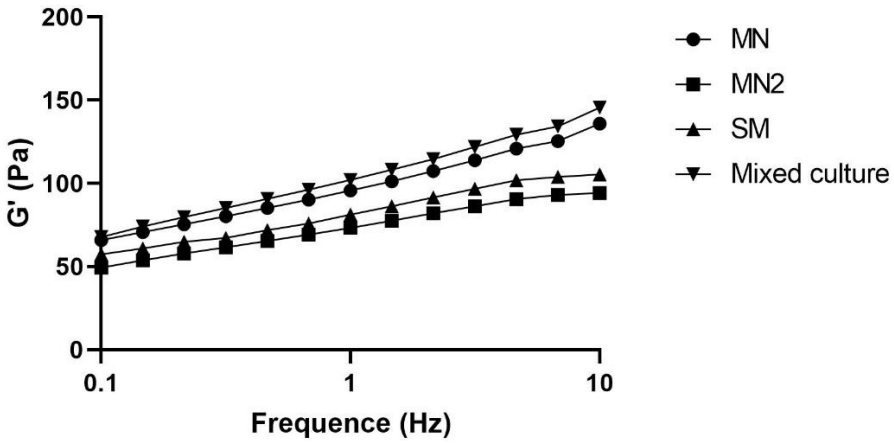



Figure 48 Dependence of G′ on frequency for fermented milk during storage on 21 days

● MN: fermented milk with *Lactobacillus paraplantarum* ■ MN: fermented milk with *Lactobacillus plantarum* ▲ SM: fermented milk with *Lactobacillus gasserri* ▼ Mixed culture: fermented milk with mixed culture

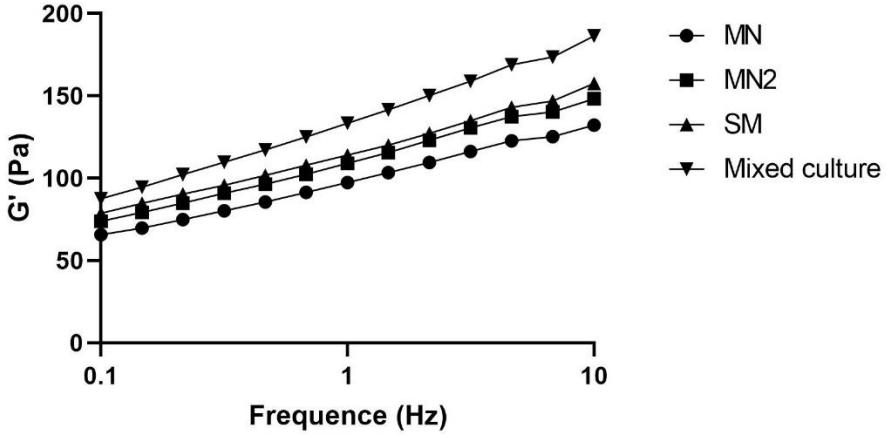



Figure 49 Dependence of G′ on frequency for fermented milk during storage on 28 days

● MN: fermented milk with *Lactobacillus paraplantarum* ■ MN: fermented milk with *Lactobacillus plantarum* ▲ SM: fermented milk with *Lactobacillus gasserri* ▼ Mixed culture: fermented milk with mixed culture

# CHAPTER 5
# DISCUSSION

Beneficial effects from the consumption of probiotics, including improvement of intestinal health by the regulation of microbiota, and enhancing the bioavailability of nutrients, reducing symptoms of lactose intolerance, and reducing the risk of certain other diseases.[138] The most essential probiotic properties must be able to survive and colonize in the human gastrointestinal tract, and thus must be able to tolerate pH and concentration of bile acids. It should not be toxic, allergic and non-pathogenic. In addition, it should be recognized by the immune system. Most probiotics are lactic acid bacteria that have been studied for a long time, and most of them are non-pathogenic[139, 140] In the present study, we proposed to isolate lactic acid bacteria from Thai fermented foods, evaluated for these parameters under laboratory conditions. Probiotic bacteria were different in beneficial effects to the host, they are strain specific. This study focused on bile salt hydrolase activity by lactic acid bacteria and determine the technological potential of lactic acid bacteria isolated from Thai fermented foods in view of their application in the fermented milk product.

Bile salt hydrolase activity of lactic acid bacteria dwell in the gastrointestinal tract is often associated with its cholesterol-lowering effects. The ability of probiotic strains to hydrolyze bile salts has often been included among the probiotic strain selection criteria. Bile acids are synthesized from cholesterol and conjugated to either glycine or taurine in the liver. Bile salt hydrolase hydrolyses conjugated to deconjugated bile salt and release free bile acid and amino acid residues. Deconjugated bile salts are few efficiently reabsorbed than conjugated bile salts, which results in the excretion of free bile acids in feces, the synthesis of new bile salt from cholesterol can potentially reduce the total cholesterol concentration in the body. The high bile salt hydrolase activity of lactobacilli might have some role in the reduction of the serum cholesterol level.

In this study, 55 lactic acid bacteria were investigated for the production of bile salt hydrolase enzyme described by direct plate assay. LAB were spotted on MRS agar supplemented with taurodeoxy cholic acid (TDCA) and incubated in anaerobic condition at 37 $^{0}$c for 72 h as showed in the Table 7, the result showed that 10 isolates from 55 LAB isolates exhibited bile salt hydrolase activity. When LAB isolate producing bile salt hydrolase were spread on MRS plates supplemented with TDCA, the taurine conjugated bile acid was deconjugated producing deoxycholic acid.[126] The deconjugation activity of LAB isolates were demonstrated in Figure 9-11 and plenty amounts of deoxycholic acid precipitated around active colonies and diffused into the surrounding medium. Three lactic acid bacteria which were MN, MN2, and SM which had the strongest bile salt hydrolase activity were proceeded to probiotic properties tests and identify the species.

Ru *et al* (2018). Reported that lactic acid bacterial strains from foods were screened for bile salt hydrolase activity. The result showed that the strains isolated from the fermented vegetables showed higher incidence of BSH-positive strains than those isolated from the fermented milk (46% versus 30%). BSH-positive strains, including *Lactobacilus hammesii*, *Lactobacilus brevis*, *Lactobacilus casei*, *Lactobacilus plantarum*, and *Lactobacilus paracasei*.[141] Similar results were reported by Liong and Shah (2005) and Kimoto *et al* (2005) showed highest deconjugation ability (BSH activity) was observed for *Lactobacillus acidophilus* ATCC 33200, and *Lactobacillus casei* ASCC 1521. Many studies suggest that the cholesterol removal mechanism *in vitro* is linked to the bile salt hydrolase activity of probiotic strains[142, 143]

In this study, 3 of total 55 isolation has the effect of producing bile salt hydrolase. Identification of species of lactic acid bacteria were performed by 16S rDNA sequencing. The 16S rDNA sequencing was determined using BLAST software compared to Eztexon-e database and phylogenetic tree analysis. The result showed that 3 lactic acid bacteria MN and MN2 isolate was closely related to *Lactobacillus paraplantarum* DMS 10667$^{T}$ and *Lactobacillus plantarum* subsp. argentoratensis DK0

$22^T$ similarity scores was 100% respectively. Isolate SM show 99.78% similarity scores to *Lactobacillus gasseri* ATCC $33323^T$.

Ahn *et al* (2003), Elkins *at al* (2001) and Dong *at al* (2012) reported the bile salt hydrolase activity of the *Lactobacillus plantarum* and *Lactobacillus gasseri.* [28, 144, 145] Many reports on hypocholesterolemic effects *in vivo* by BSH-producing lactic acid bacteria have led to increased attention in maintaining cholesterol levels in normal people or the possible applications for hypercholesterolemia individuals.[146, 147]

Microorganisms to be applied as probiotic must be able to survive and colonize in the human gastrointestinal tract. In order to reach active and viable enough through GIT, they should be resistant to acid, lysozyme and bile.[148] The ability of bacteria to survive in the gastrointestinal tract is an important probiotic property. The pH of excreted HCl in stomach is 2.0, but the presence of food raises the pH value to 3.0. In the study, 3 isolates were tested for acid tolerance. The low pH tolerance of lactic acid bacteria was determined at pH values 2.0, 3.0 and 4.0. The results show that all isolates could survive after incubation for 3 h at pH 3.0 and pH 4.0 but at pH 2.0 all isolate were not survive.

Good probiotic sources should withstand at least pH 3.0. Generally, there is a reduction in probiotic count, as they were exposed to pH 2.0 and pH 3.0 and the count is fairly constant at pH 4.0 and MRS control (Table 9.). These results are in agreement with the study of Guo *et al* (2010). They reported that the viable cell counts of all lactic acid bacteria were significantly affected by the low acidity, especially at pH 2.5 and 2.0. [149, 150]According to Zavaglia *et al* (2002) reported that hydrochloric acid (HCl) found in the human stomach is a strong oxidizer. Therefore, able to oxidize many important biomolecules in the cell and destroy them while it is undergo reduction. As demonstrated by Sultana *et a*l (2000) and Chan and Zhang (2005), lactic acid bacteria members such as *L. acidophilus*, *L. plantarum* mainly could not survive in low pH environment as these cells were proven to be vulnerable at pH 2.0 and below.[151-153] Many studies also confirmed that exposing to acid with 2 after 3 hours incubation caused reduction in viable counts of lactic acid bacteria intensively.[154, 155] Therefore,

result in table 9 indicates the strong inhibition on the viable bacteria numbers at pH 2.0 was well supported.

After the bacteria have passed the stomach, they enter the upper intestinal. The relevant physiological concentrations of human bile range from 0.3% to 0.5%. The presence of bile salt hydrolase (BSH) in probiotics perform them more tolerant to bile salts[156] Bile tolerance test of 3 isolates lactic acid bacteria result showed that all isolates could survive after incubation for 3 h at ox gall concentration 0.3. At ox gall 0.8% the trend cell decreased, with higher inhibition of growth seen as the bile concentrations increased MRS broth as control for all experiments and it recorded the highest growth. (Table 10).

Overall in the results, bile did not inhibit the growth of the bacteria completely as even when subjected to 0.8% of bile, there is still a high number ~ log $10^6$-$10^7$ CFU/mL. The selective probiotic strains proved to present an excellent quality of bile tolerance. Another important factor is the bile salt hydrolase (BSH) activity which accounts for the bile salt resistance. They can be producing bile salt hydrolase enzyme. Lactic acid bacteria with positive for BSH activity implying that it might survive better in the host GI tract, because bacterial BSH mediates deconjugation of bile salts which improves the intestinal viability of probiotics.[157] Study by Suskovic *et al* (2001) showed that some strains where BSH hydrolase conjugated bile, thus reducing its bactericidal effect.

Microbial adhesion to the surface of the intestinal mucus and epithelial cells is one of the most important characteristics of probiotics. It influences the antimicrobial and immunomodulation effects that depend on colonization of the gastrointestinal tract by probiotic bacteria. The physical and chemical properties of the surface of bacterial cells depend mainly on its hydrophobicity.[158, 159] In order to gain information on the structural properties of the cell surface of lactic acid bacteria that are responsible for aggregation and adhesion, its hydrophobicity/hydrophilicity.

In the study, 3 isolates were tested for cell surface hydrophobicity the result showed the percent hydrophobic value of MN, MN2 and SM was 64.26%,57.80% and

50.0% respectively. Isolate MN showed maximum affinity toward xylene (64.26%) than other isolates. The hydrophobic reaction of bacteria plays an important role in the adhesion and formation of biofilm.[22(160)] However, the observation by Yadav *et al* (2016) studies potential probiotic *Lactobacillus i*solated from indigenous fermented beverage Raabadi, consumed during summers in Haryana and Rajasthan regions of India found that *Lactobacillus plantarum* RYPR1. They showed values of hydrophobicity highest 79.13%.[(161)]

Currently, bacteria species clinically relevant are the focus for the study on the presence and dissemination of antibiotic resistance.[(162)] The important requirement for probiotic strains is that they should not carry transmissible antibiotic resistance genes.[25] [(163)]In the present study regarding antibiotic resistance, the isolates showed resistance to nalidixic acid and streptomycin. Also, maximum susceptibility of all isolates was observed against chloramphenicol, tetracycline, ampicillin and gentamicin.

Kirtzalidou *et al (2011),* Fukao *et al* reported that amongst lactobacilli a high resistance to aminoglycosides such as kanamycin, streptomycin and gentamicin. Likewise, previous studies have also reported high resistance to nalidixic acid. [(164-166)] Ammor MS *et al* (2007), Coppola *et al* (2005) have reported that lactobacilli are usually sensitive to ampicillin and generally susceptible to antibiotics inhibiting the synthesis of proteins, such as chloramphenicol, erythromycin, clindamycin and tetracycline, and lactobacilli are usually resistant to most inhibitors of nucleic acid synthesis, including nalidixic acid.[(167, 168)] Innate resistance of probiotics to some antibiotics suggests their use for the purpose of preventing and therapeutic in controlling of intestinal infections, especially when combined with the use of antibiotics in therapeutic.[(169)]

Analysis of hemolysis is very important prerequisites for safety because many organisms are able to synthetic exotoxins that induce the partial or total lysis of human or animal cells.[(170)] As per safety concerns, a potential probiotic bacterium should not

cause lysis of red blood cells in the body. In this study, all the tree isolates were incapable of exhibiting hemolysis on the agar media containing 5% human blood. Which means selected lactic acid bacteria isolated do not exhibit pathogenicity and are safe for consumption. This is in agreement with other study reports of lactic acid bacteria confirming that they are non-hemolytic in nature.[171] Likewise, Escamilla-Montes *et al* (2015) and Padmavathi *et al* (2018). No hemolysis activity was found in probiotics lactic acid bacteria.[172, 173]

Antimicrobial activity against pathogens is another important feature that must be considered in selecting potential probiotic strains for maintaining a healthy microbial balance in gastrointestinal tract. In the study investigations, the antimicrobial activity of probiotics was tested against the pathogenic bacteria 12 strains. The result showed that isolates MN2 presented non-antimicrobial activity against pathogens observed from showed non-inhibit zone against all pathogens. However, isolate MN showed low inhibition against *Shigella dysenteria* and isolate SM showed low inhibition against *Vibrio parahaemolyticus* and *Shigella dysenteria.* (Table13). Probiotic bacteria were different in beneficial properties beneficial effects to the host, they are strain specific. Based on the results of this study, all isolates presented no antimicrobial activity against pathogens property.

Similar study of Marie *et al* (2009). reported study antimicrobial activity of *L. paraplantarum* KNUC25 isolate from Kimchi found that *Lactobacillus paraplantarum* KNUC25 showed least antimicrobial activity against *Shigella dysenteria* and *Salmonella paratyphica* ATCC11511.[174]

Previously*, Lactobacillus gasseri* has been reported antimicrobial to produce a number of bacitracins, with the most well-characterized being gassericin A from *Lactobacillus gasseri* LA39, which was isolated from infant feces.[175]

In this study, 3 isolates of LAB were having the strongest bile salt hydrolase activity applied to determine the technological potential of lactic acid bacteria isolated

from Thai fermented foods in view of their application in the fermented milk product. Milk was inoculated with 3 probiotic strains of single culture and mixture culture at a ratio of 1;1;1 the initial concentration of bacteria was $10^9$ CFU/ml Incubated at 40°C until pH of milk reaches 4.6 . Quality parameters of fermented milk during storage at 5°C will be evaluated every 7 days for 28 days.

Present fermentation times (to reach pH 4.6) of probiotics for the prepared fermented milks (initial pH of milk was 6.74) (table14). In the study, fermented milks with MN, MN2, SM and Mixed culture used fermentation times (to reach pH 4.6) at 18, 19, 21 and 16 hr respectively. Fermented milk with mixture culture used the shortest fermentation time. Long fermentation times (21 hr) were observed in SM. Variance in fermentation time could be due to differences in the ability of lactic acid bacteria to grow and ferment milk.

Similar results were reported by Mani-López *et al* (2014).[176] Donkor *et al* (2007) Damin *et al* (2008) have also reported that probiotic bacteria have a poor acidification performance in milk compared with common yogurt starter cultures.[177, 178]

Viability of probiotic in fermented milk product until the time of consumption is the most critical factor of these products. The Food and Drug Administration (FDA or USFDA) has also suggested that the minimum probiotic count in a probiotic food should be at least $10^6$ CFU/mL.[174] The numbers of probiotic lactic acid bacteria used in this study of the fermented milk during the experiment are shown in Table15. Figures22 present changes in Lactic acid bacteria counts during fermentation and storage of fermented milk. Inoculation was initial ~ $10^9$ CFU/ml end of the fermentation (at pH = 4.5) counts 24 hr after fermentation were 9.2, 9.3, 9.4 and 8.3 log CFU/mL for MN, MN2, SM and Mixture culture, respectively (Figure 22.). Fermented milk with MN, MN2, SM and Mixed culture decreased by log 1.28 1.33 1.13 and 1.42, respectively cycles after 28 days of storage. Mixed culture decreased by 2.34 log of 28 days storage. Observed that after 28 days of storage the fermented milk with SM has the highest number of cells

count. After 28 days fermented milk with MN MN2 SM and Mixed culture observed that cell counts declined but counts were still >$10^6$ CFU/mL at the end of the storage.

The results of the present study suggested that fermented milk with Mixed culture may be inhibited by the presence of other species within a multi-species probiotic. It may be that these different species inhibit each other.[179] A loss of probiotic viability occurred during storage of the fermented milk, which could be related to acidity, the presence of oxygen in the media. When probiotic cells are in an environment with a low pH (<4.5), more energy is required to maintain the intracellular pH, resulting in the lack of ATP for other important functions and thus causing cell death. The continuous exposure to oxygen under acidic conditions during storage is the main cause of the reduction of probiotics counts.[180, 181] From the standpoint of consumer's health benefits, the selected probiotic cultures must maintain their viability and functionality during the product storage period.[182] Counts above $10^6$ CFU/mL are considered values potentially functional for probiotics or populations of about $10^7$-$10^9$ CFU in the daily portion of the products.[177, 183] Thus, the fermented milk could be considered probiotic for 7, 14, 21 and 28 days.

In study of Gueimonde *et al.* (2004) 14 commercial fermented milks, reported counts from $10^7$ to $10^9$ CFU/mL after 30 days of storage .[184] Similar reports Mortazavian *et al* ( 2007) and Damin *et al.* (2008) observed a decrease around 2 log of *Lactobacilus acidophilus* in yogurt after 28 d of storage.[178, 185] Overall, the viability of lactic acid bacteria depends on strain type, storage conditions, and culture mixture.[176]

pH in fermented products during Storage. In this study, pH values of fermented milks decreased during storage 28 days. Over the total storage period, the most changes in pH were seen in fermented with MN, MN2, SM and Mixed culture, which it decreased from 4.6 in day 1 to 4, 4, 3.98 and 4.05, respectively in 28 days of during storage. Fermented milk that include isolate SM presented the highest acidity values in storage for 28 days. Fermented milk with SM presented the highest acidity values. Figure23 shows the tendency of fermented milk pH to decrease during storage.

Declining pH of fermented milk may be metabolic behavior of microorganisms due to the continuous production of acids by lactic acid bacteria present in these products.[186] Gueimonde *et al* (2004) analyzed 14 commercial fermented milks and observed pH values around 3.9 to 4.2. [186]Panesar and Shinde (2012) obtained pH of 4.03, 3.91 after 21days of strorage (5°C) in yogurts products fermented with *Streptococcus salivarius* subsp. thermophilus and *Lactobacillus delbrueckii* subsp [187] This results are similar to these reports, confirm the residual acidification during storage.

The low pH can be considered the main factors detrimental to the viability of probiotics in the stomach. The pH of the stomach mainly in the range of 2.5 to 3.5 but may be as low as pH 1.0 or pH 2.0 at higher gastric juice secretion rates.[188, 189] Food normally remains in the stomach for 2–4 h The effect of acidic conditions on the viability of lactic acid bacteria in fermented milk is shown in Table 17-18. At pH3 fermented milk of all isolate 28 days of storage show significant decrease. On 1 to 28 days of storage fermented milk with MN, MN2, SM and Mixture culture decreased by log 0.79, 0.85, 1.17 and 0.95 respectively (Table 17). At pH2 fermented milk of all isolate not survive on 14, 21, and 28 days of storage (Table 18). At pH3, fermented milks of all isolates maintained cell count $10^6$ - $10^7$ on 28 days of storage, which maintained recommended levels at least $10^6$ CFU/mL. At pH2.0, fermented milks of all isolate maintained cell count $10^6$ on 14 days of storage. On 21 to 28 days of storage cell cannot survive. When compare pure culture at pH2 fermented milk of all isolates can survive on 4 days of storage but pure culture cannot survive.

Having passed through the acidic gastric environment, the probiotics are face surviving in the small intestinal environment, where they are exposed to bile salts. Bile tolerance 0.3 % and 0.8% of fermented milks with probiotic lactic acid bacteria 3h exposure was demonstrated in Table 19-20.

At ox gall 0.8% on 28 days of storage fermented milk with Mixture culture survive cell count is less than the recommended count $10^6$ CFU/mL may be due to

inhibition of multi-species probiotic within fermented milks product. The survival rate of fermented milk of all isolates on 28 days storage trend tolerance in ox gall 0.3% and 0.8% can tolerance than pure culture. In this study probiotic strains proved to exhibit an excellent quality of bile tolerance. Another important factor is the bile salt hydrolase (BSH) activity which report for the bile salt resistance. Sahadeva *et al* (2011) reported *lactobacilus* strains with BSH hydrolyse conjugated bile, thus reducing its bactericidal effect.[154, 190]

Overall, milk demonstrated influence on improving probiotic viability in the presence of bile and acid. Ranadheera et al (2012) reported that fat contents in yogurt and ice cream may have provided protection to probiotics by reducing their exposure to acid and bile.[191] Recent studies have shown that some LAB in fermented fruit and vegetable are resistant to acid and bile, similar to the LAB found in animal sources, so these products can be used as a suitable carrier for probiotics. Differences in the storage temperatures and storage time may have caused variations in the acid tolerance of the probiotics.[192, 193]

The existence of BSH activity probiotic lactic acid bacteria in fermented milk was demonstrated in Table 21. All fermented milk showed existence of maximum BSH activity in fermented milk during storage 28 days. On 28 days of storage fermented milk with Mixture culture presented the least existence of BSH activity. Due to fermented milk with Mixture culture the count of viable cells decreases causing a decrease in BSH production.

Lactobacilli is often used in human consumption products and can be found in probiotics in baby food, milk, and many types of pharmaceutical preparations. Bile salt hydrolase enzymes of many species of LAB species play an important role in the metabolism of the hosts. Over the past decades, probiotics bacteria with BSH activity were used to alleviate cholesterol levels in humans and animals. Fermented milk with probiotic have bile salt hydrolase activity may be an alternative way to reduce

cholesterol. [194] In the present study, in view of their application in milk product. It was found that probiotic isolate could maintain BSH activity in fermented milk products.

Similar study of Champagne *et al* (2016) reported that when adding L*actobacillus* with BSH activity to the fermented product found that could producing BSH activity in fermented products.[195] Changlu *et al* (2019) reported that *Lactobacillus plantarum* CAAS showed the greater BSH activity causing a significant decrease in the serum cholesterol levels in hamsters. [196]

*Lactobacillus parapalntarum*, *Lactobacillus plantarum* and *Lactobacillus gasseri* showed the greater BSH activity in this study, which suggests that this strain was able to hydrolyze conjugated bile salts to release amino acids and free bile acids. The free bile acids are less soluble and less efficiently reabsorbed from the intestinal lumen than their bile conjugated forms which clause increase fecal bile acid excretion levels. To replace the lost bile acids, more new bile acids would be synthesized from cholesterol in the hepatic witch results decrease in the serum cholesterol levels in blood.[25, 197] In addition, the greater of bile salt hydrolase ability also promoted survival of probiotic in the gastrointestinal tract observed from result viability of probiotics in acid and bile (Table17 -19).

Fermented milk product is organised as a concentrated dispersion of protein particles, aggregates and clusters[198]. Texture is an important attribute of fermented milk product quality.

Fermented milk gel structure is the result of casein aggregation by pH reduction and denatured whey proteins. In the study, the firmness values of fermented milk with probiotic during 28 days storage presented in Table22. All fermented milk firmness significantly increased during cold storage. Fermented milk with Mixture culture exhibited the highest values of firmness. A higher firmness of fermented milk has also been related to a longer fermentation time.[178] In this study, fermented milk with long fermentation time may displayed greater firmness. The increase in firmness during storage could be related to further pH reduction, which may cause the structure of the

gel to shrink, with a consequent elevation of gel strength.[199] Similar study of Sah *et al* 2016. report that yogurt with probiotic exhibited the highest values of firmness during storage 28 days[200]

Rheology properties of foods is the study of the deformation and flow of food materials. Rheological of the fermented milk depend on its microstructure and physicochemical interactions between the structure element casein micelles. In this study, storage modulus (G'), which determined solid-like property of materials, significantly increased at higher frequencies of fermented milk with MN, MN2, SM and Mixed culture. Fermented milk with Mixture culture have the highest G' compared with fermented milk with MN MN2 and SM.

Syneresis is the separation of the liquid phase from the curd. In fermented milk syneresis is undesirable. The loss of whey is a measure of the level of the gel that is collapsed and is an indicator of poor quality and stability. In this study, syneresis is the separation of the liquid phase from the gel. In fermented milk syneresis is undesirable. There was a significant reduction in syneresis during the 28 days of refrigerated storage (figure44) Fermented milks with MN and Mixed culture exhibited significant increases in % syneresis on day 7 and fermented milks with MN2 and SM significant increases in % syneresis on day 14. The lowest and highest syneresis values were fermented milk with Mixed culture and fermented milk with SM, respectively. A significant ($p < 0.05$) increase in syneresis was observed during cold storage, which was more pronounced in fermented with SM.

Lowering of pH during storage likely resulted in contraction of the casein network and consequently greater whey expulsion was similar to that reported by Amatayakul *et al* (2006) report that fond syneresis 9-14% of yoghurt during 28 days of storage. [198, 201]

# CONCLUSIONS

In the present study, 55 LAB isolates were isolated from fermented foods and only three isolates were selected on the basis of their strongest bile salt hydrolase activity namely MN, MN2 and SM. These 3 isolates were identified using 16S ribosomal DNA sequence analysis similar as *Lactobacillus paraplantarum*, *Lactobacillus plantarum* and *Lactobacillus gasserri*.

Three strains exhibited good resistance to gastrointestinal condition (pH, 3; bile salt, 0.3% and 0.8%). For cell surface hydrophobicity, MN, MN2 and SM show moderate hydrophobicity. Three isolates showed resistance to nalidixic acid and streptomycin. Also, maximum susceptibility of all isolates was observed against chloramphenicol, tetracycline, ampicillin and gentamicin. All the three isolates were incapable of exhibiting hemolysis

In view of their application in milk product it was found that *Lactobacillus paraplantarum*, *Lactobacillus plantarum* and *Lactobacillus gasserri* could maintain BSH activity in fermented milk products. Therefore, with the properties of good probiotics these strains could be potentially used in functional food and health products especially where cholesterol reduction by bile salt hydrolase in food is the main target. Further *in vivo* study is necessary to prove the hypercholesterolemic effect of the *Lactobacillus paraplantarum*, *Lactobacillus plantarum* and *Lactobacillus gasserri* by producing bile salt hydrolase.

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