CHARACTERIZATION OF PROBIOTIC LACTIC ACID BACTERIA WITH ANTIOXIDANT POTENTIAL *IN VITRO* AND *IN VIVO* AND THEIR APPLICATION IN PROBIOTIC PRODUCTS

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การศึกษาคุณลักษณะของโพรไบโอติกแบคทีเรียกรดแลกติกที่มีศักยภาพต้านอนุมูลอิสระในหลอดทดลองและสัตว์ทดลองและการประยุกต์ใช้ในผลิตภัณฑ์โพรไบโอติก

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PORNTIPHA VITHEEJONGJAROEN

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BY

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The aims of this study are (1) to investigate the antioxidative activities of lactic acid bacteria (LAB), isolated from healthy infant feces; and (2) to develop a probiotic yoghurt with antioxidative activities. The viable cells, cell-free supernatant and intracellular cell-free extract of MSMC36-9, MSMC37-3, MSMC37-4, and MSMC83 at a concentration of 1×109 CFU/mL exhibited antioxidant activities including 2,2-diphenyl-1-picryl-hydrazyl (DPPH) radical scavenging capacity, hydroxyl radical scavenging capacity and an intracellular free radical level on Caco-2 cells. The evaluation of the probiotic properties of four LAB isolates showed a high survival rate in gastrointestinal conditions, and a substantial adherence to Caco-2 cells. MSMC36-9, MSMC37-3, MSMC37-4 were assigned to *Lactobacillus paracasei* while MSCM83 was assigned to *Bifidobacterium animalis* based on their 16S rRNA sequence analysis and physiological properties. Among the four LAB isolates, *B. animalis* MSMC83 was the strong antioxidant activity and the highest potential for probiotic properties. Therefore, *B. animalis* MSMC83 was selected to be analyzed for antioxidant activity in oxidative stress rats induced by D-galactose. The results showed that *B. animalis* MSMC83 significantly reduced fasting blood glucose, total cholesterol, and triglyceride. Interestingly, the administration of *B. animalis* MSMC83 significantly increased the antioxidant enzymes, consisting of superoxide dismutase (SOD), catalase, and glutathione peroxidase (GSH-Px) in serum, while the content of malondialdehyde (MDA) decreased. Moreover, liver tumour necrosis factor-alpha (TNF-alpha) significantly decreased in probiotic groups when compared with the D-galactose model group. The analysis of gut microbiota revealed that *B. animalis* MSMC83 could recover the microbiota diversity after induced D-galactose. Further, *B. animalis* MSMC83 was selected to use in the production of probiotic yoghurt. The fermentation of *B. animalis* MSMC83 in combination with *Streptococcus thermophilus* and *Lactobacillus bulgaricus* at the ratio of 1:1:1 in milk for the development of yoghurt was performed, and the microbiological and physicochemical properties of yoghurt were investigated while in refrigerated storage. The results showed that fermentation time for yoghurt with added *B. animalis* MSMC83 was significantly shortened. The viable cell counts remained stable throughout the storage period. *B. animalis* MSMC83 could be used as probiotic culture in the development of functional foods for preventing and controlling the diseases associated with oxidative stress.

Keyword : probiotics, lactic acid bacteria, antioxidant, oxidative stress, yoghurt

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CHAPTER I
INTRODUCTION

Probiotics have come into play in medical and health care, animal feeds and foods and other agricultural products. Probiotics help in strengthen and enhance health of hosts. The definition of probiotics by the Food and Agricultural Organization of the United Nations/World Health Organization (FAO/WHO) are “live microorganisms which when administered in adequate amounts confer a beneficial health effect on the host”.[1] In the human body, probiotic microorganisms are found living in the gastrointestinal tract as normal microbiota. They are also found in many food products such as dairy products, fermented foods and food supplements. These probiotics are diverse and have different beneficial properties depending on the specific characteristics of each strain. Those beneficial properties are immunomodulation, anti-pathogenic activity, anti-allergy, reduction of inflammatory bowel disease and chronic disease symptoms, anti-cancer, reduction of hyperlipidemia and antioxidant activity.[2-4]

The major probiotic microorganisms belong to lactic acid bacteria (LAB), named as such because most of its members convert lactose and other sugars to lactic acid. LAB are gram-positive and non-spore forming, and are able to grow under microaerophilic or anaerobic conditions. The most commonly utilized LAB as probiotic microorganisms include *Lactobacillus*, *Bifidobacterium* and *Enterococcus*.[5] Furthermore, lactic acid bacteria are normal microbiota of the human gastrointestinal tract. Several species have been isolated from humans, animals, vegetables, dairy and fermented food products.[6-9] Survival of these bacteria in the gastrointestinal tract is considered a key function as probiotics. The survival is based on their resistance to low pH and/or bile and their temperature growth ranges. Additionally, they can adhere to intestinal tissues and colonize in the human gastrointestinal tract.[10] Because of the industrial importance of LAB, they further obtained Generally Recognized As Safe (GRAS) status. Health promoting characteristics and contribution to gut microbiota of probiotics make these microorganisms desirable for use in the production of dairy and

other food products.[11] LAB in fermented foods are primarily responsible for imparting health benefits. The benefits of LAB have been shown in a number of host physiological responses including immunomodulation[12], anti-carcinogenic[13], anti-tumour activity[14], reduction of cholesterol[15] and increase in antioxidant activities.[11]

One factor that underlies the pathogenesis of several chronic health problems including diabetes mellitus, cancer, liver disease and several degenerative chronic diseases is an increase in free radicals. Free radicals are derived either from normal essential metabolic processes in the cells or stimulation form extrinsic factors. The molecules of free radicals are single or unpaired electrons, which can react with other free radicals by either donating an electron to or accepting an electron from other molecules. They can also interact simultaneously with various biomolecules around them. Therefore, it results in abnormalities and damage to the cells.[16] The free radicals are generally divided into 3 groups including reactive oxygen species (ROS), reactive nitrogen species (RNS) and reactive chlorine species (RCS).[17] ROS are the most important from all free radicals. Three of the major ROS including superoxide radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO^{\cdot}$) are normal products of metabolism, that are generated continuously in the mitochondria of growing cells.[18] Hydroxyl radical is the most dangerous free radical in the group of ROS. The superoxide anion radical is most likely to trigger free radicals and also have the ability to interact with many other biomolecules.[19] These ROS present a contrast in their function. On one hand, they prevent diseases by assisting the immune system, mediating cell signaling and playing an essential role in apoptosis. On the other hand, they destroy important molecules in the cells and may play a role in the pathogenesis of many diseases.[20]

Normally, the body prevent and block free radicals using antioxidants. The antioxidants resist oxidation-reduction in the cells and inhibit free radicals from the chain reaction.[21] In human cells, the primary antioxidants, necessary for all oxygen metabolizing cells, consist of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and catalase. Superoxide dismutase eliminates toxicity of superoxide anions and prevents the superoxide anions mediated reduction of iron followed by hydroxyl

radical generation. Catalase converts hydrogen peroxide to oxygen and water. Glutathione peroxidase is an enzyme that protects hemoglobin from oxidative degradation in red blood cells. Additionally, glutathione peroxidase requires glutathione reductase (GR) and glucose-6-phosphate dehydrogenase (G-6-PD) in the glutathione system, which enhances the function of the GSH-Px.[22, 23] Thus, antioxidants control the level of free radicals. When body is stimulated by various intrinsic and extrinsic stress factors, free radicals are generated. If free radicals are higher than antioxidants, the body remains in oxidative stress. Oxidative stress is the term that refers to the imbalance between free radical generation and the activity of the antioxidant defenses. Severe oxidative stress causes cell damage and death, and has been implicated in numerous human diseases, such as diabetes mellitus, heart disease, rheumatoid arthritis, inflammatory bowel diseases, colon cancer, Alzheimer's disease and aging.[18, 20, 24, 25] Excessive free radicals in cells can react with different biomolecules, resulting in the breakdown of the DNA strand, transforming proteins and the formation of lipid peroxide. The lipid peroxidation induce the release of toxics and reactive aldehyde metabolites such as malondialdehyde (MDA).[26, 27] As a result, the body acquires necessary antioxidants to help control free radicals and prevent diseases.[19] Additionally, the role of free radical reactions in human pathogenesis has become an intense interest. Scientists, therefore, have researched enormously on the roles and types of antioxidants in protecting the body against damages caused by free radicals. At present, the public has a great interest in health and the knowledge of healthy food is linked in functional food development, especially the use of LAB probiotics.

Probiotic products are a processed product that add live probiotics in the right amount and concentration to be able to benefit the body. Probiotics are traditionally added to food products like yogurts and other fermented dairy products, juices, and other fermented foods.[28] Among probiotic food products, dairy products are the first commercialized products and still consumed in larger quantities than other products. Some strains of LAB are able to synthesize exopolysaccharides. In respect to food industry, exopolysaccharides are used as natural thickeners, emulsifiers, gelling agents

and stabilizers.[29] The use of LAB in foods has technological significance in the production of several fermented dairy products and largely responsible for the structural, mechanical, and physicochemical properties of the products.[30] Therefore, the active ingredients of fermented milk have a synergistic effects between LAB and the biogenic components generated following the reaction with LAB. Additionally, the texture and stability are major criteria of food quality in order to provide optimum structural quality to design new and attractive foods.[31] Thus, a diverse variety of dairy products with different properties can be obtained using different technologies and starter cultures. Lactic fermentation of milk plays a major role in the manufacturing of fermented dairy products. Important features of the bacterial starters are rapid acidification, microbial preservation of the milk, formation of specific flavours, texturing capacities.[32-34] For example, the milk supplemented with probiotic LAB had significant effects on acidification, texture, and microbiological stability of fermented milk.[35] Fermented milk made from *B. animalis* ssp. *l*actis BLO4 in coculture with *S. thermophilus* TA040 gave the most constant rheological behavior and the best cell viability during cold storage compared to *L. delbrueckii* ssp. *bulgaricus* LB340 and *L. acidophilus* LAC4.[36] In general, *S. thermophilus* and *L. delbruekii* subsp. *bulgaricus* have been used in traditional yoghurt as starter cultures.[37] In addition, *L. acidophilus* and *B. bifidum* was used to make ice cream and found that the survival was slightly decreased during 17 week of frozen storage at –29°C.[38]

Over the past decades, scientists have focused on the importance of LAB for use in biotherapeutics, especially LAB with antioxidant activity. From the previous study, lactic acid bacteria from different food samples possess free radical scavenging activities ranging from 14.7-50.8% after 24-72 hour fermentation.[39] The *B. longum* and *L. delbrueckii* had the ability to scavenge free radical by 70.4-75.1% using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical assay, which examines antioxidant effect of hydrogenation. Besides, the malondialdehyde production in intestine 407 cell lines decreased when cell membrane was treated with LAB.[40] Moreover, 10 *Lactobacillus* strains were assessed for their protective effects against radical induced

oxidative damage in HepG2 cells, and resulted in an inhibition of oxidative radiation-induced 2', 7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) in cellular antioxidant assay.[41] In aging mouse following D-galactose administration, after oral ingestion of *L. plantarum* MA2, it was found that MDA levels decreased and total antioxidant capacity increased with increasing SOD and GSH-Px activities in the serum and liver tissues.[42] Additionally, the *B. animalis* 01 administration were significantly increased the antioxidant enzymes in serum of aging mice, while mice's MDA content were reduced both in serum and liver tissues.[43] In clinical trial, the probiotic yogurt consumption increased erythrocyte SOD and GSH-Px activities and total antioxidant status, while the serum MDA concentration significantly decreased in type 2 diabetic patients.[44] In addition, administration of two *Lactobacillus* strain supplement in the athletes following intense physical activity, which induced oxidative stress, resulted in an increase in plasma antioxidant levels. From athletes feces after probiotic administration, it was found that both strains were recovered from the fecal samples in the control group, while group of probiotics were significantly increased.[45] The previous studies showed that, antioxidant activity of lactic acid bacteria differed in each species. However, there is not much data on the anti-oxidative ability of lactic acid bacteria available to date.

Finding and developing antioxidants is essential to protect cells from free radicals and prevention of the progression of many chronic diseases. Moreover, there is evidence to show that LAB probiotics have significant antioxidant abilities, thus raising interest in the LAB probiotic's antioxidant properties. In this study, lactic acid bacteria isolated from healthy newborn feces and fermented foods and will be investigated for their antioxidant potential in chemical assays using hydroxyl radical and 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay. After that, selected lactic acid bacteria will be further investigated for antioxidant activity using intracellular free radical level in cell lines with oxidative stress including human colon carcinoma (Caco-2) cell line. Then, strains which have antioxidant activities will be chosen for the study of general probiotic properties including acid and bile tolerances and adherence

properties. They will also be characterized by 16S rRNA gene sequencing. Additionally, the best probiotic lactic acid bacteria with antioxidant properties in chemical and cellular assays and exhibited maximum potential probiotic will be chosen for determination of antioxidant activities in serum including superoxide dismutase (SOD), catalase, glutathione peroxidase (GSH-Px), malondialdehyde (MDA) and inflammatory in liver tissues including tumor necrosis factor-alpha (TNF-alpha) in an animal model. Further, this study will monitor the persistence and changes in type and amount of intestinal microbiota in animals treated. Finally, application of selected probiotic lactic acid bacteria in yoghurt form of fermented milk products will be performed. Properties of yoghurt product including pH profile, firmness, syneresis, and rheological properties will be investigated.

Hypothesis

Specific strains of probiotic lactic acid bacteria are able to increase antioxidant and free radical scavenging activities *in vitro* and *in vivo*.

Objectives

1. To search for antioxidant activities of lactic acid bacteria on the reduction of hydroxyl radical ($HO^{\cdot}$) and 2,2-diphenyl-2-picrylhydrazyl (DPPH) free radical in chemical assays.

2. To investigate antioxidant activities of selected lactic acid bacteria on the reduction of intracellular free radical level in human colon carcinoma (Caco-2) cell line.

3. To investigate the acid and bile tolerances and adherence properties of selected lactic acid bacteria.

4. To identify the selected probiotic lactic acid bacteria by 16S rRNA gene sequencing.

5. To investigate the antioxidant activities of selected probiotic lactic acid bacteria on the increase of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), catalase and decrease of malondialdehyde (MDA) in animal model.

6. To investigate the anti-inflammatory property of selected probiotic lactic acid bacteria on the decrease of tumor necrosis factor-alpha (TNF-alpha) in animal model.

7. To monitor intestinal microbiota in animals treated with selected probiotic lactic acid bacteria.

8. To investigate chemical and physical properties of yoghurt fermented with selected probiotic lactic acid bacteria.

CHAPTER II
LITERATURE REVIEW

1. Background and definition of probiotics

The word ‘‘probiotics’’ was initially utilized as an antonym of the word ‘‘antibiotic’’. The term is derived from the Greek words pro and biotos and being translated ‘‘for life’’. The probiotic was first used by Kollath in 1953 which he suggested the term as all organic and inorganic food complexes. In 1965, the term was modified again by Lily and Stilwell. They defined as “substances secreted by one microorganism, which stimulates the growth of another”.[46] In relation to food, probiotics are described as ‘‘viable preparations in foods or dietary supplements to improve the health of humans and animals’’.[2] A recent official definition of probiotics by World Health Organization (WHO) joint committee with Food and Agricultural Organization of the United Nations (FAO) are “live microorganisms which when administered in adequate amounts confer a beneficial health effect on the host”.[1]

In the human body, probiotic microorganisms are found living in the gastrointestinal tract as normal microbiota. They are also found in many food products such as dairy products, fermented foods and food supplements.[47-49]

Probiotic products are processed for a various and diverse of dietary purposes such as yogurt, cheese, and fermented foods. The benefits of using probiotics are well documented to prevent and maintain healthy bowel function. In addition, probiotics have the ability to treat digestive disorders and other disease. Also, probiotic consumption affects changes in the gut microflora and has several health-promoting properties such as immunomodulation, anti-pathogenic activity, anti-allergy, reduction of inflammatory bowel disease and chronic disease symptoms, anti-cancer, reduction of hyperlipidemia and antioxidant activity.[2-4] Additionally, the applicability criteria of probiotics are used in the production of functional food and in the preservation of food products.[50]

According to these definitions, groups of probiotic microorganisms are different in terms of featured characteristics, genus and species. The genus and species of

microorganisms considered as probiotics are shown in table 1.[51] Interestingly, the largest group of probiotic microorganisms is lactic acid bacteria (LAB) such as *Lactobacillus, Bifidobacterium Enterococcus* and *Lactococcus*. Two strains, *Lactobacillus* and *Bifidobacterium* are widely studied.

Table 1 Microorganisms considered as probiotics.

Genus	Species
Lactic acid bacteria group	
Lactobacillus	*L. casei, L. delbrueckii, L. fermentum, L. gasseri, L. johnsonii, L. lactis, L. paracasei, L. plantarum, L. rhamnosus, L. reuteri,*
Bifidobacterium	*B. adolescentis, B. animalis, B. bifidum, B. infantis, B. lactis, B. longum, B. pseudolongum, B. thermophilum*
Enterococcus	*E. faecium, E. faecalis*
Lactococcus	*L. garvieae, L. lactis*
Streptococcus	*S. thermophilus, S. salivarius*
Pediococcus	*P. pentosaceus*
Non-lactic acid bacteria group	
Bacillus	*B. cereus, B. clausii, B. coagulans, B. licheniformis, B. subtilis*
Escherichia	*E. coli* Nissle
Propionibacterium	*P. freudenreichii*
Aspergillus	*A. oryzae*
Saccharomyces	*S. boulardii, S. cerevisiae*

2. Lactic acid bacteria

The major probiotic microorganisms belong to lactic acid bacteria (LAB), named as such because most of its members convert lactose and other sugars to lactic acid as the major metabolic end-product of carbohydrate fermentation.[52] LAB are gram-positive and non-spore forming, and are able to grow under microaerophilic or anaerobic conditions. The most commonly utilized LAB as probiotic microorganisms include *Lactobacillus* and *Bifidobacterium*.[5] They contain many curing and preventive effects such as production of antimicrobial content for pathogen antagonism, reduction of alleviate lactose intolerance complications, reduction of cancer risk, cholesterol reduction both direct and indirect, immunomodulation and increase in antioxidant activities.[11-15] In part of industry, various species of LAB have been used for the production of fermented milk products in many countries for thousands of years.[50]

2.1 *Lactobacillus*

Bacteria belonging to the genus *Lactobacillus* are members of the LAB. Lactobacilli are non-spore-forming, gram-positive rods, thin rods varying in length from long to short and non-motile. Most species of lactobacilli are facultative anaerobes growing in either the presence or absence of an anaerobic environment. There are more than 20 species of lactobacilli that have been used for treating and preventing a wide variety of diseases and conditions such as *L. acidophilus*, *L. bulgaricus*, *L. rhamnosus* GG, *L. plantarium*, *L. reuteri*, *L. salivarius*, *L. casei*, *L. johnsonii*, and *L. gasseri*.[53, 54] They are an important part of the normal human bacterial microbiota naturally found in the oral cavities, gastrointestinal (GI) tract, vaginas of female and animals.[6-9, 55, 56] Several species of *Lactobacillus* are widely distributed in environment mainly rich in carbohydrates, such as plant, animal feeds, silage and manure. In food industry It has a history of safe use in human food and nutrition.[57] They have an important role in the manufacture of fermented vegetables, wine sausages and have been used commercially for the production of sour milks, cheeses, and yogurt.[55, 56]

2.2 *Bifidobacterium*

First discovered in 1900 by Henry Tissier, *Bifidobacteria* were first isolated from the feces of healthy breast-fed infants and named *Bacillus bifidus*.[58] Subsequently, *Bacillus bifidus* was later re-named *Lactobacillus bifidus* because of its similarity to Lactobacilli. They were classified as a separate genus in 1974 which are now known as *Bifidobacterium bifidum*.[59, 60] Members of the genus *Bifidobacterium* are generally characterized as Gram-positive, non-spore-forming, non-motile, non-gas producing, catalase-negative and anaerobic. They produce acetic and lactic acid from carbohydrates without the generation of CO_2. In addition, their morphology is generally curved or Y-shaped like a branch. They present in the gastrointestinal tract within days of birth, especially in infants, they are thought to be the best marker of intestinal health.[61] For example, *B. bifidum*, *B. breve*, *B. infantis*, *B. lactis*, *B. thermophilum*, *B. longum* and *B. pseudolongum* are part of human and animal intestinal microbiota. Some species are used by the food and drink industries. Interestingly, different strains give many health benefits such as regulation of intestinal microbial hemostasis, inhibition of pathogenic bacteria, modulation of immune responses system, reduction of atopic dermatitis, promotion of brain function, reduction of free radical and bio-convention of diary compound into bio-active molecules.[62-65] Therefore, the properties are species strain specific.

3. Criteria of probiotic strains

The criteria of screening for probiotic strains are safe, manufacturing, administration. Additionally, they need to adhere to intestinal tissues and colonize in the human gastrointestinal tract. Because of the industrial importance of LAB, they further obtained Generally Recognized As Safe (GRAS) status. Survival of these bacteria in the gastrointestinal tract is considered a key function as probiotics which is based on their resistance to low pH and/or bile and their temperature growth ranges.[10] The characteristics recommended to be used for selection of probiotic strains are present in table 2.[66, 67] The first step in selection of a probiotic LAB strain is to examine

of its taxonomic classification together with the indication of the origin, habitat and physiology of the strain. All these characteristics have important result on the selection of the novel strains. Based on these selection criteria, they are many test *in vitro* experiments to screen microorganisms on their potential as probiotic strains. Functional properties of probiotics can be done with animal and human trials.[68] On the other hand, the same probiotic species, strains, and subspecies vary by habitat, environment, and many other characteristics.[69-71]

Table 2 Criteria for probiotic strains.

Desirable characteristics of an ideal probiotic microorganisms
Generally Recognized As Safe (GRAS) status
Non-pathogenic and non toxic
Acid and bile tolerance
Adhesion to mucosal and epithelial surface
Prevention of pathogen adhesion and colonization
Production of antimicrobial substances
Ability to modulate immune responses
Resistance to technologic process
Viability in the gut and during storage

4. Health benefits of probiotics

Recently, probiotics have come into play in medical and health care, animal feeds and foods and other agricultural products. Probiotics help in strengthen and enhance health of hosts. The most important beneficial effects of probiotics include the prevention of diarrhea, constipation, enhancement of antibacterial activity, anti-inflammatory, reduction of cholesterol and increase in antioxidant activities.[11-15] However, the effects of probiotics are strain specific.[72]

4.1 Protection of diarrhea

Diarrhea is a common symptom and a major cause of global health problem. The most often reason for diarrhea is the presence of bad bacteria, yeast, virus or toxins in the gut. One potential to prevent this side effect is the use of probiotic. Previous research shows that when used in healthy children, some probiotic species and strains are safe and result in preventing and treating infectious diarrhea.[73] In addition, the use of *L. rhamnosus* GG can reduce the risk of traveler's diarrhea and cured patients with recurrent *Clostridium difficile* infection. Also, numerous clinical trials show that some strains can improve the outcome of intestinal infections by reducing the duration of diarrhea.[74-78] Therefore, the use of probiotics reduce dependence on antibiotics which is expensive, and is well tolerated, even for prolonged use.

4.2 Improvement of lactose utilization

Lactose is a sugar found in milk and it is digested by lactic enzymes created in the body. In human who are lactose intolerance, the body cannot produce lactic enzyme to break down the sugar and absorbed in the small intestine. The microorganisms in the gastrointestinal tract used this sugar in acid and gas generation. As a result, diarrhea caused by water enters the intestine and bowel movement is faster. In human study with lactose intolerance was reduce in breath hydrogen and was reduce in symptom scores from supplementation with probiotics in yogurt cultures. Because probiotics help digest lactose and use lactose as a substrate for lactic acid production.[79, 80]

4.3 Cholesterol-lowering

Previous research has shown that probiotics have the potential to lower cholesterol through different mechanisms such as assimilation of cholesterol, binding of cholesterol to the cell surface, interference with the formation of micelle for intestinal absorption, and secretion of bile salt hydrolase (BSH) which deconjugates bile acids. *Lactobacillus* and *Bifidobacterium* strains are found to produce BSH. The BSH makes bile salts insoluble and is excreted in the feces through deconjugate of bile acids. This mechanism is to reduce serum cholesterol by increasing *de novo* synthesis to replace lost bile acid by using.[81, 82] In the *in vitro* system, probiotics can remove

cholesterol from culture media.[83] This has been reported that BSH has the same effect. In addition, two strains of probiotics have potential of cholesterol assimilation.[84] In clinical trial, probiotic fermented yogurts were administered in healthy human. The results showed that the group consuming probiotic yoghurt were significantly decreased total cholesterol and LDL, compared with the placebo group.[85]

4.4 Anti-cancer

Cancer is an important public health problem, with more than 10 million patients worldwide and is on an increasing trend. About half of those are dead, although surgery, chemotherapy and radiation therapy are used as a means of treating cancer. Evidence from various sources supports the assumption that the relation between diet and cancer may be due to an imbalance of the intestinal microflora in body.[86] *In vitro* studies showed that several probiotics LAB may help prevent initiation of colon cancer. Briefly, probiotics reduce tumor cell viability, suppress induced carcinogenesis in cancer cells and bind potent mutagenic metabolic compounds.[87] Additionally, LAB has shown anti-neoplastic properties in a variety of cancer cell lines of both human and animal origin. The study of the effect of fermented milks on colon cancer cells showed a decrease in growth of the cells. However, probiotics for the prevention or treatment of cancer in humans is needed to be studied more.[88]

4.5 Reduction of inflammation

Acute and chronic inflammation is the body's immune system in telling us to pay attention to our health, because the inflammation is a natural part of the immune response to sickness or infection. Probiotics can affect the immune system in the host, especially anti-inflammatory. It is the ability of specific probiotic strains to significantly decrease tumor necrosis factor-alpha (TNF-alpha) which the production of messengers called pro-inflammatory cytokines.[89, 90] In hypersensitive human of clinical studies, probiotics are able to stimulate the immune system and lower the inflammatory responses.[91] Therefore, probiotic LAB are more known for its ability to protect and treat inflammatory bowel diseases in adults, and improve the immune system without any adverse effects in both *in vitro* and animal models.[92]

4.6 Increase in antioxidant activities

Over the past decades, scientists have focused on the importance of LAB for use in biotherapeutics, especially LAB with antioxidant activity. From the previous study, probiotic LAB were found as promising microorganisms for the protection and control of several free radical-related disorders.[93] Reduction of free radicals was demonstrated that the viable cells and intracellular cell free extracts (ICFE) of each *Lactobacillus* had antioxidant activity by different assessments of the ability to absorb free radicals of specific oxygen.[94] In animal, the effects of oral administration of probiotic LAB were to increase superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) activities and to decrease malondialdehyde (MDA) level in serum. Similarly, activity of hepatic catalase was increased and hepatic MDA levels were decreased.[95] Additionally, the inhibiting plasma lipid peroxidation was evaluated from mix-culture probiotics. Thus, probiotics can different protect plasma lipids from levels of oxidation and LAB-fermented dairy products can also be a potent to resist oxidative stress.[96] In ageing mice that administration of probiotics can reduce aging wrinkles caused by oxidative stress and improve expression of antioxidant enzyme.[97] In the human gastrointestinal tract of healthy volunteers, it was found that the glutathione red-ox ratio was significantly decreased when administered with probiotics fermented goat's milk.[98] Therefore, probiotics have antioxidant activity through the mechanism of scavenging or generation of free radical and stimulating the creation of antioxidants.

5. Free radical

One factor that underlies the pathogenesis of several chronic health problems including diabetes mellitus, cancer, liver disease and several degenerative chronic diseases is an increase in free radicals. Free radicals are derived either from normal essential metabolic processes in the cells or stimulation form extrinsic factors. The molecules of free radicals are single or unpaired electrons, which can react with other free radicals by either donating an electron to or accepting an electron from other molecules.[16] The most important free radicals in many disease states are hydroxyl

radical ($HO^{\cdot}$), superoxide anion radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and nitric oxide radical ($NO^{\cdot}$). They can also interact simultaneously with various biomolecules around them such as nucleic acids, proteins, carbohydrates, and lipids.[99] Therefore, it results in abnormalities and damage to the cells. The free radicals are generally divided into 3 groups including reactive oxygen species (ROS), reactive nitrogen species (RNS) and reactive chlorine species (RCS).[17] ROS are the most important from all free radicals.

6. Reactive oxygen species (ROS)

The reactive oxygen species (ROS) are a molecule that resulting from the partial reduction of oxygen. They consist of radical and non-radical oxygen species. The process of mitochondrial oxidative phosphorylation has always produced the ROS. Additionally, environmental stresses such as ultraviolet light, certain drugs, cigarette smoking, and metal toxicity, promote the production of ROS due to disruption of cellular homeostasis.[100] Three of the major ROS including superoxide radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO^{\cdot}$) are normal products of metabolism, generated continuously in the mitochondria of growing cells.[18] ROS is created in the reaction. Haber-Weiss, which finally became a hydroxyl radical. In the first step, ferric ions are iron ions, reacts with superoxide ions to form hydrogen peroxide. Then, the second step is the Fenton reaction by hydrogen peroxide in Fenton oxidation reaction with ferric, resulting in a ferrous ion and hydroxyl radical (Figure 1).[101]

$$Fe^{3+} + {}^{\bullet}O_2^- \longrightarrow Fe^{2+} + O_2. \qquad (1)$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^- + {}^{\bullet}OH. \qquad (2)$$

Figure 1 Step of reactive oxygen species.

Reference: Lipinski B. Hydroxyl radical and its scavengers in health and disease. Oxid Med Cell Longev. 2011; 2011: 809696

Hydroxyl radical is the most dangerous free radical in the group of ROS. The superoxide anion radical is most likely to trigger free radicals and also have the ability to interact with many other biomolecules.[19] These ROS has different functions and the ability to react. Low amounts of ROS in cells can aid the immune system, stimulating cell signaling mediators in apoptosis. On the other hand, excess ROS destroy important molecules in cells and may play a role in the pathogenesis of many diseases.[20] The effects of ROS on the pathogenesis of the disease have not been shown to directly cause damage to the molecule. However, there is evidence that ROS indirectly causes disease.

7. Generation of free radicals

The formation of free radicals can derive from endogenous (within the body) or exogenous (outside the body) as show in Figure 2.[102] The exogenous source is from environmental sources including ultraviolet light, certain drugs, cigarette smoking, air pollutants, ozone, and industrial chemicals.[103, 104] Similarly, the compounds from food, water, drugs and air are sources of free radical. On the other hand, the endogenous source of free radical is from metabolic processes in the human body such as cellular respiration in mitochondria, inflammation, phagocytosis and cell injury. These are produced in the body by four different mechanisms.

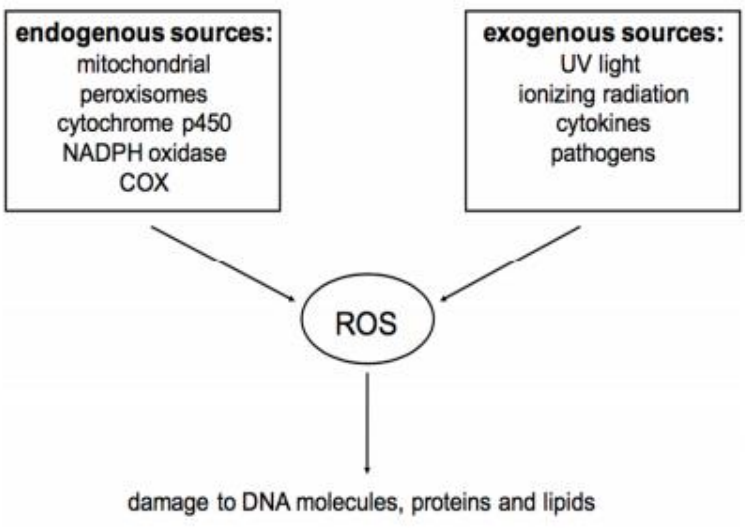

Figure 2 Sources of reactive oxygen species (ROS).

Reference: Waris G, Ahsan H. Reactive oxygen species: role in the development of cancer and various chronic conditions. J Carcinog. 2006; 5: 14.

The first mechanism is cellular respiration in mitochondria. Mitochondria normally consumes oxygen in the process and convert it to water which produces the universal energy molecules adenosine triphosphate (ATP). Nevertheless, unwanted by-products including H_2O_2, and $OH^{\bullet-}$ are naturally produced due to incomplete reduction of oxygen. Each day, regular metabolism is expected to produce over 20 billion oxidized molecules. Human must respire to get oxygen to live, but it may be harmful and causes damaging effects on the human body from excessive amounts of these free radicals occurred when we are exposed to air pollution and cigarette smoke.[105]

The second mechanism can be described as white blood cells destroy pathogens such as bacteria, virus, parasite and toxics by using free radical. The most commonly used free radical are $NO^{\cdot}$, $O_2^{\cdot-}$ and H_2O_2 (Figure 3).[106] These free radicals attack molecules in order to get the electrons again, and damage the molecules. These molecules losses its electrons and become free radicals to begin a chain reaction. When this process starts, it causes tissue damage from cell disruption.[107]

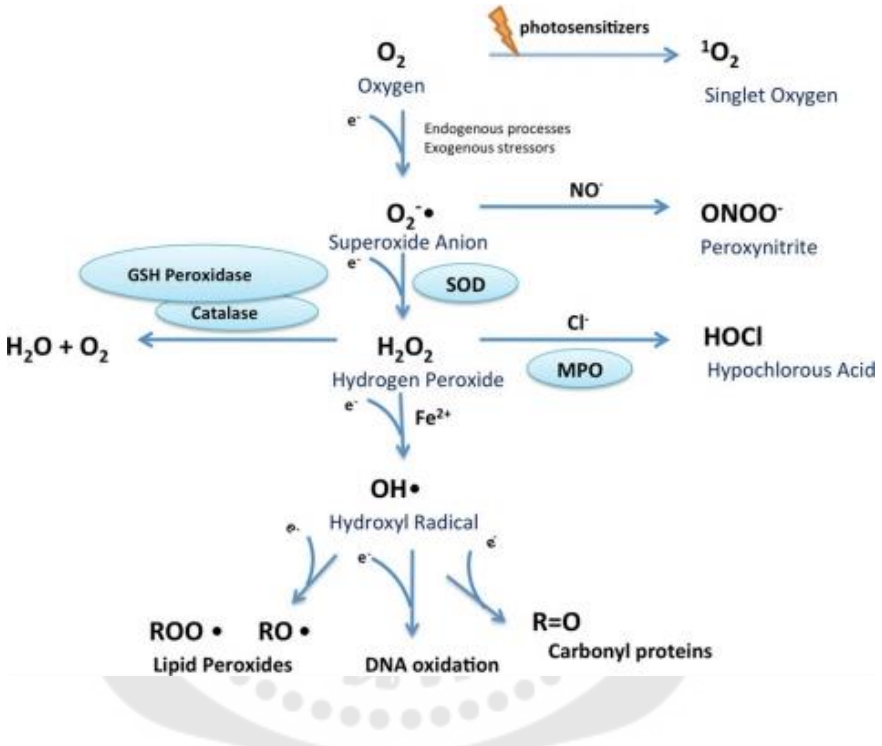

Figure 3 Generation of reactive oxygen species.

Reference: Chen L, Hu JY, Wang SQ. The role of antioxidants in photoprotection: a critical review. J Am Acad Dermatol. 2012; 67(5): 1013-24

The third mechanism is the production of H_2O_2, a by-product of the degradation of fatty acids and other molecules called peroxisomes. Polyunsaturated fatty acids, which have multiple double bonds, are strongly associated with the production of free radicals. Oxidizing leads to an increase in the production of free radicals in the form of lipid peroxide radicals.[105]

The final mechanism is the body's primary defenses against toxins in food by the enzyme in the cells called cytochrome P450. These enzymes produce oxidant by-products to prevent damage from toxic chemicals such as drugs and pesticides.[99]

8. Antioxidant

In the nineteenth century, antioxidants have been used in the rubber industry, which helped to reduce degradation and optimize the vulcanization process. Later, antioxidant were defined as "any substance that, when present at low concentrations compared with that of an oxidizable substrate, significantly delays or inhibits oxidation of that substrate" by Halliwell and Gutteridge in 1995.[108] But later they redefined in 2007 as "any substance that delays, prevents or removes oxidative damage to a target molecule".[109] While Khlebnikov and colleagues defined them as "any substance that directly scavenges ROS or indirectly acts to up-regulate antioxidant defenses or inhibit ROS production" in the same year.[110]

Antioxidants are important to human life. Normally, the body prevents and blocks free radicals using antioxidants. The antioxidants resist oxidation-reduction in the cells and inhibit free radicals from the chain reaction.[21] The human antioxidant system is divided into two major groups, enzymatic antioxidants and non-enzymatic antioxidants. Enzymatic antioxidants are comprised of limited number of proteins such as superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and catalase. Non-enzymatic antioxidants can derive from dietary sources including ascorbic and lipoic acid, polyphenols and carotenoids.[111]

In recent years, antioxidants have been studied extensively and agreed that "the use of antioxidants in foods is good", which is expected to reduce the risk of many diseases such as diabetes mellitus, atherosclerosis, neurodegenerative diseases, cancer and other diseases associated with ageing.[112]

8.1 Enzymatic of antioxidant

In the body, the primary antioxidants, necessary for all oxygen metabolizing cells, consist of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and catalase.[22, 23] These enzymes play an major role in balancing free radicals in host. They systematic mechanisms are shown in Figure 4.[113, 114]

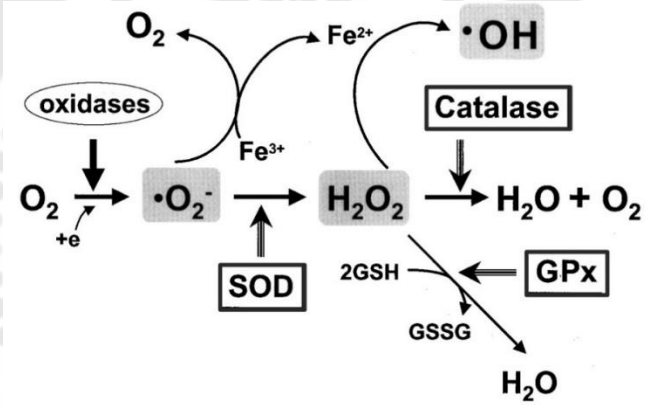

Figure 4 Mechanism of primary antioxidants enzyme.

Referance: Griendling KK, FitzGerald GA. Oxidative stress and cardiovascular injury: Part I: basic mechanisms and in vivo monitoring of ROS. Circulation. 2003; 108(16): 1912-6

Superoxide dismutase eliminates toxicity of superoxide anions and prevents the superoxide anions mediated reduction of iron followed by hydroxyl radical generation. These enzymes have either a manganese, copper, or zinc cofactor. SOD enzymes are the fastest enzymes, and they are highly exposed to free radicals with greater number and detoxifying activity. SOD levels have been associated with

inflammation, neurodegenerative diseases, metabolic diseases, liver diseases. Superoxide anions have a pro-inflammatory role and induce lipid peroxidation, and recruitment of neutrophils to sites of inflammation and ageing. Additionally, SOD plays a role in preventing mitochondrial DNA damage by participating in the mitochondrial repair processes. SOD is used to treat diseases such as arthritis, inflammatory diseases, inflammatory bowel disease, cancer, and prevent side effects from long-term exposure to smoke and radiation. It is also an option in cosmeceuticals to reduce wrinkles, and hyperpigmentation on the face and protect against harmful UV rays. [115, 116]

Catalase converts hydrogen peroxide to oxygen and water using iron as a cofactor. Catalase consists of four subunits, which each subunit containing a ferric (Fe^{3+}) heme group bound to its active site. Catalase is found extremely in peroxisomes and heart mitochondria. When there is a large amounts of hydrogen peroxide expression, they result in the diffusing of the cytosol to the methionine. Because catalase enzymes are impermeable to the mitochondrial membrane, they are the most important scavenger in the cytosol. These enzymes detox the free radical from the action of SOD. Catalase enzymes are found in high amounts in the cell and scavenge the hydroxide peroxide. They can destroy millions of hydrogen peroxide per second. The reduction of catalase was associated with oxidative stress, inflammation and number of diseases such as diabetes mellitus, atherosclerosis, colitis, hepatitis, and cancer.[117]

The last primary enzyme is glutathione peroxidase. These enzymes convert hydrogen peroxide to water and oxygen similar to catalase. Additionally, these enzymes are to protect hemoglobin from oxidative degradation in red blood cells. Additionally, glutathione peroxidase requires glutathione reductase (GR) and glucose-6-phosphate dehydrogenase (G-6-PD) in the glutathione system, which enhances the function of the GSH-Px. These enzymes have six isoforms consist of cytosolic (GSH-Px-1, cGSH-Px), gastrointestinal (GSH-Px-2, giGSH-Px), plasma (GSH-Px-3, pGSH-Px), and the phospholipid hydroperoxides peroxidase (GSH-Px-4, phGSH-Px).[118] The GSH-Px-1 is an important isoform and is found in the cytoplasm and mitochondrial matrix. While

the GSH-Px-4 is responsible forrepairing damage caused by mitochondrial oxidation and preventing mitochondrial ATP by reducing lipid hydroperoxides, alkyl peroxides, and fatty acid hydroperoxides. The GSH-Px interact with pro-inflammation activation by TNF-alpha which prevent transport of lipid peroxides and oxidative damage and maintain the mitochondrial oxidative-phosphorylation. Previous studies showed that these enzymes are able to decline of malondialdehyde (MDA) which are lipid peroxidation aldehydes that can stimulate the release of toxins and react with other substances.[26, 27]

9. Oxidative stress

Normally, antioxidants control the level of free radicals. When body is stimulated by various intrinsic and extrinsic stress factors, free radicals are generated. If free radicals are higher than antioxidants, the body remains in oxidative stress. Oxidative stress is the term that refers to the imbalance between free radical generation and the activity of the antioxidant defenses. Excessive free radicals in cells react with other biomolecules, resulting in DNA breakdown, protein transformation, polysaccharide degradation, and lipid peroxide formation. Interestingly, lipid peroxidation is a chain reaction from unsaturated fatty acids (cell membrane components) oxidized with free radical and is the cause of various pathological conditions. These processes cause damage to the membrane cells. Its effect causes the receptors to be damaged, which in turn affects the regulation of various organs. When the hydrogen atoms are transferred from the fatty acid molecule to free radicals, it begins a severe chain reaction. The end products of lipid peroxidation induce the release of toxics and reactive aldehyde metabolites such as malondialdehyde (MDA).[26, 27] Oxidative stress can have many biological effects, such as apoptosis and viral proliferation. Additionally, oxidative stress increases inflammatory response, resulting in excessive tissue damage. The most oxidation stress sensors are genes transcription factors such as nuclear factor-κB (NF-κB). Nuclear transcription factor NF-κB translocates from cytosol to nucleus. This leads to the translate control of many gene expression associated

with inflammation. On the other hand, increasing tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), lipopolysaccharide, viral infection, and ultraviolet light can stimulate the formation of free radicals, which have NF-κB act as the second messenger. Therefore, NF-κB is able to bind to DNA in a process controlled by redox reactions.[119, 120] In addition, this signaling activates the transcription of several genes that code for natural antioxidants such as the enzymes superoxide dismutase (SOD).[121]

Severe oxidative stress causes cell damage and death, and has been implicated in numerous human diseases, such as diabetes mellitus, heart disease, rheumatoid arthritis, inflammatory bowel diseases, colon cancer, Alzheimer's disease and aging.[122] As a result, the body acquires necessary antioxidants to help control free radicals and prevent diseases.[19] Additionally, the role of free radical reactions in human pathogenesis has become an intense interest. Scientists, therefore, have researched enormously on the roles and types of antioxidants in protecting the body against damages caused by free radicals.[18, 20, 24, 25]

10. Action modes of probiotic LAB in antioxidation

Scientists have focused on the importance of LAB for use in biotherapeutics, especially LAB with antioxidant activity. Recent studies have shown that antioxidant mechanisms depend on the species of probiotics.[123]

10.1 Scavenging activity ability

The first mode is a synergistic action of deoxidizing and the presence of ion-bound chelators involved in free radical formation.

Their activities on the hydrogen destruction process lead to the restoration of the core functions of antioxidants. Previous studies showed that lactic acid bacteria from different food samples possess free radical scavenging activities ranging from 14.7-50.8% after 24-72 h fermentation.[39] Antioxidant *L. rhamnosus* GG had significantly longer survival times in H_2O_2 compared to non-antioxidant *Lactobacillus* strains. In addition, intracellular free extracts (ICFE) of the two strains of *Lactobacillus* have different specificities in their free radical scavenging activity.[124] The studies of DPPH

radical scavenging activities of *Bifidobacterium* and *Lactobacillus* exhibited 71.3% and 70.4% for viable cells and 72.6% and 75.1% for ICFE, respectively.[40] Similarly, *L. fermentum* has the effect of scavenging hydroxyl radical, superoxide anion radical and DPPH radicals.[95] While the 19 LAB strains were studied by the metal ion chelating ability and also showed Fe^{2+} or Cu^{2+} ion chelation. Six *L. bulgaricus* strains is highly Cu^{2+} chelating abilities.[11] In *B. animalis* 01, the culture supernatant showed more than 70% in hydroxyl radical and superoxide anion radical scavenging activities.[125] Besides, the malondialdehyde production in intestine 407 cell lines decreased when cell membrane was treated with LAB.[40] Therefore, many LAB strains were highly antioxidant through chelating ions. Therefore, many LAB strains are high in antioxidants by chelating ion. [126, 127] Degradation of hydroperoxides to produce ROS by changing metal ions. The effects of oxygen scavengers and ion chelators are to reduce free radicals and reduce the chain reaction of free radicals.

10.2 Antioxidant enzymes system

In human, probiotics have antioxidant enzymatic systems. SOD, GSH-Px and catalase are the major antioxidant enzymes. The SOD is the most important scavenger in both cytosol and mitochondria but GSH-Px and catalase are the most important scavengers in the cytosol. Another mechanism of probiotics is that it can produce antioxidant enzymes, which are direct neutralization of intestinal oxidants. In addition, enzymes can also stimulate the immune system by reduction of inflammation and prevention of cytokine-induced oxidative stress.[128] In aging mouse following D-galactose administration, after oral ingestion of *L. plantarum* MA2, it was found that MDA levels decreased and total antioxidant capacity increased with increasing SOD and GSH-Px activities in the serum and liver tissues.[42] Additionally, the *B. animalis* 01 administration significantly enhanced the activities of antioxidant enzymes in serum of aging mice, while mice's MDA content was reduced both in serum and liver tissues.[125] In mice with Crohn's disease receiving LAB, SOD and catalase enzymatic activities in the gut increased, and inflammation in the small intestine reduced.[129] Normally, LAB are generally considered to be catalase-negative or produce very small amounts of

catalase. On the other hand, the administration of *L. plantarum* ZLP001 in weaning piglets were enhance the concentration of SOD, GSH-Px and catalase while reduced the concentration of MDA.[130] Additionally the expression of SOD and GSH-PX- gene were significantly up-regulated in the intestinal cells treated with *Lactobacillus*. Similarly, the many *Lactobacillus* isolates significantly up-regulated expression of catalase gene.[131] Research in type 2 diabetic patients receiving probiotic products consists of *Lactobacillus* and *Bifidobacterium* found that SOD, GSH-Px activities and total antioxidant were increased.[44] Oral administration of *L. casei*, *L. acidophilus* significantly down-regulated interleukin-6 (IL-6), TNF-α and IL-1β (pro-inflammatory cytokines) and up-regulated IL-17, IL-4, and IL-10 (anti-inflammatory cytokines). Additionally, they significantly decreased oxidative stress in rat induced arthritis.[132] Therefore, probiotics can stimulate the production and expression of genes involved in the host's antioxidant system and reduce inflammatory cytokines.

10.3 Regulating microbiota

The newborn is exposed to the bacteria in the mother's vagina through natural birth or to the mother's skin through caesarean section, resulting in the development of the baby's microbiome.[133] Neonatal gut bacteria are found in facultative anaerobes such as *Enterobacteria, Bifidobacterium*, *Streptococci*, *Bacteroides*, and *Clostridium*.[134, 135] The important modulation of the intestinal microflora in the newborn depends on the amount of environment and the genetic factor in the production of microorganisms in the intestine.[136] In general, bacteria are found in the human intestine at approximately 10^{14} bacteria, which is more than 10 times the body cells. These bacteria are highly differentiated by up to 400-500 species.[137] The intestinal microbiota has several mechanisms to protect the host from pathogens such as competitive exclusion, including occupation of attachment sites, consumption of nutrient sources, and production of antimicrobial substances (Figure 5).[138, 139]

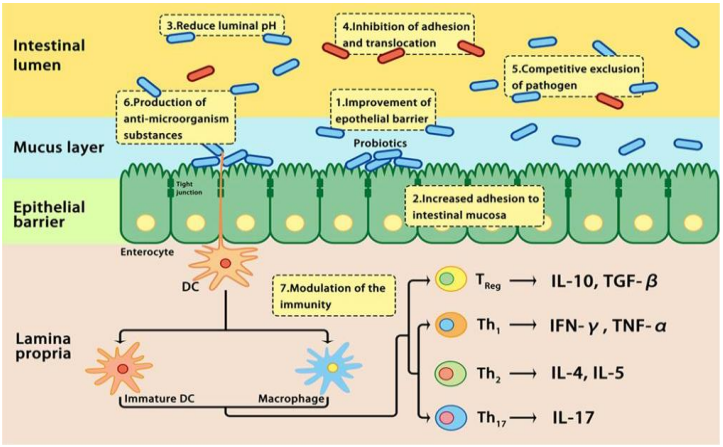

Figure 5 Mechanisms of action of probiotics to protect the host from pathogens.

Reference: Zhang M, Sun K, Wu Y, Yang Y, Tso P, Wu Z. Interactions between intestinal microbiota and host immune response in inflammatory bowel disease. Front Immunol. 2017; 8: 942.

The abnormal intestinal microbiota showed bad bacteria proliferate too much, are cause significant oxidative stress. The bacterial community is another mechanism that affects the homeostatic redox in the host.[140] Although there are studies of probiotics related to microbiota, small intestine and gastrointestinal disease, but the studies on the antioxidant activity of microorganisms in the intestine are relatively minor. *Lactobacillus* and *Bifidobacterium* producing lactic acid, acetic acid, and propionic acid, result in reduction of intestinal pH and suppression of the growth of different pathogens.[141, 142] Recently, a study has reported that high-fat diet (HFD) -fed mice have an association with oxidative stress, which affects the change of gut microbiota.[143] The research found that. *L. johnsonii* BS15 administration changed the gut microbiota in HFD-induced oxidative stress mice as similar to normal mice.[144] Similarly, intestinal tract of obese mice fed with food supplemented with *L. curvatus* HY7601 and *L. plantarum* KY1032 has been changed.[145] In clinical trial, administration of two *Lactobacillus* strain supplementation in the athletes following intense physical activity to induce oxidative stress resulted in an increase in plasma antioxidant levels. From athletes feces after

probiotic administration, it was found that both strains were recovered from the fecal samples in the control group, while groups of probiotics were significantly increased.[45]

11. Role of probiotics in food industries

Probiotics are popular as an ingredient in health supplements under the general term " functional foods". The viable probiotics in probiotic products have the right amount and concentration to perform beneficial functions. In large commercial, probiotic foods are a group of very interesting functional foods. This leads the industries to introduce different probiotics into their products and creating a new generation of "probiotic health foods" to claim health benefits.

In early human history, preservation of milk and meat by fermentation were used. Then LAB has been used to ferment vegetables in China in the third century. In addition to being investigated for health benefits, LAB is currently being developed to improve retention quality and nutritional value.[146] Especially, the LAB are used to produce various fermented dairy products, such as yogurt, cheese, kefir and many others. Examples of commercial probiotic strains and products are present in table 3.[147]

Table 3 Examples of commercial probiotic strains and products.

Species / strain	Brand name	Producer	Claimed effect in humans/animals
B. animalis ssp. *lactis* BB-12	BB-12	Chr. Hansen	Reduction in *S. mutans* in mouth, IBS amelioration in a multispecies trial
B. animalis ssp. *lactis* HN019 (DR10)	Howaru Bifido	Danisco	Reduced prevalence of atopy and eczema in the first 2 years of life
B. breve Yakult	Bifiene	Yakult	Ulcerative colitis amelioration
B. infantis 35624	Align	Procter & Gamble	Irritable bowel syndrome treatment
B. longum BB536	BB536	Morinaga	Treatment of allergy, especially Japanese cedar pollinosis
L. acidophilus DDS-1	DDS-1	Nebraska Cultures	Alleviation of traveller's diarrhea; vitamin production
L. brevis KB290	LABRE	Kagome	Improvement of bowel movement, enhances NK activity and IFN activity
L. casei Shirota	Yakult	Yakult	Alleviation of acute diarrhea
L. paracasei St11	Lactobacillus fortis	Nestlé	Natural defense/immune system, gut health
L. johnsonii NCC533	LC1 range	Nestlé	Immunomodulation
L. rhamnosus GG	Vifit & others	Valio	Immune stimulation, alleviates atopic eczema, prevents diarrhea in children and many other types of diarrhea
L. acidophilus NCFM & *B. bifidum* BB-12	Florajen3	American Lifeline, Inc.	Reduction of *C. difficile*–associated disease (CDAD)

Among probiotic food products, fermented milks are the first commercialized products and still consumed in larger quantities than other products of because fermented milks are the ideal vehicle for delivering probiotics to the human intestines. In addition, these products have a suitable environment that supports the growth and survival of probiotics.[46, 147-149] The major species of LAB potentially used in fermented milk are *Lactobacillus, Enterococcus,* and *Bifidobacterium* such as *L. bulgaricus*, *E. faecium* and *B. animalis*. The use of LAB in foods has technological significance in the production of several fermented dairy products and largely responsible for the structural, mechanical, and physicochemical properties of the products.[30] Therefore, LAB and the metabolites produced by LAB affect the physiochemical properties of fermented milk. In designing new and interesting foods, textures and stabilizers are the main criteria for selecting the quality of fermented milk.[31] Using the technology and the starter cultures, dairy products were diversified and had different properties. Additionally, the viability of bacteria is important. It is suggested that probiotic products should contain an adequate amount of live bacteria (at least 10^6-10^7 CFU/g).[150] A minimum viable probiotic count of 10^6 CFU/g at the time of consumption in fermented milks is recommended.[151] Lactic fermentation of milk plays a major role in the manufacturing of fermented dairy products. Important features of the bacteria cultures are rapid acid production from carbohydrates and adequate in raw materials. Some LAB strains can produce ethanol, exopolysaccharides and certain enzymes that cause structural changes.[32-34] For example, the milk supplemented with probiotic LAB had significant effects on acidification, texture, and microbiological stability of fermented milk.[35] Fermented milk made from *B. animalis* ssp. *lactis* BLO4 in co-culture with *S. thermophilus* TA040 gave the most constant rheological *behavior* and the best cell viability during cold storage compared to *L. bulgaricus* LB340 and *L. acidophilus* LAC4.[36] In general, *S. thermophilus* and *L. delbruekii* subsp. *bulgaricus* have been used in traditional yoghurt as starter cultures.[37] In addition, *L. acidophilus* and *B. bifidum* was used to make ice cream and found that the survival was slightly decreased during 17 week during frozen storage at –29°C.[38]

CHAPTER III
MATERIALS AND METHODS

Materials

1 deMan Rogosa Sharpe (MRS) media (HiMedia, India)

2 Anaerobic jar (Mitsubishi, Japan)

3 Brilliant green (Sigma-Aldrich, USA)

4 Iron(II) sulfate heptahydrate ($FeSO_4$) (Merck, Germany)

5 H_2O_2 3.0% (w/v) (Merck, Germany)

6 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma-Aldrich, USA)

7 2', 7'-dichlorodihydrofluorescein diacetate (DCFDA; Sigma-Aldrich, USA)

8 Tris-HCl (Merck, Germany)

9 Triton X-100 (Amresco, USA)

10 human colon adenocarcinoma cell line (Caco-2 cells; ATCC HTB-037)

11 Dulbecco's modified eagle's minimal medium (DMEM) (Gibco-Invitrogen, USA)

12 Fetal bovine serum (Gibco-Invitrogen, USA)

13 Penicillin-Streptomycin (Gibco-Invitrogen, USA)

14 0.25 % Trypsin-EDTA (Gibco-Invitrogen, USA)

15 Trypan blue (Gibco-Invitrogen, USA)

16 Dimethyl sufoxide (DMSO) (Sigma-Aldrich, USA)

17 Hemocytometer (Hausser Scientific, USA)

18 Counter (Fisher Scientific, USA)

19 24-well tissue culture plates (Corning, USA)

20 pH meter (Thermo Scientific, USA)

21 Incubator (Selecta, Spain)

22 Centrifuge (Sartorius Stedim, Germany)

23 Spectrophotometers (Shimadzu, Japan)

24 Microscope (Olympus, Japan)

25 Laminar flow hood (Nuaire, USA)

26 PCR authorized thermal cycler (Eppendrof, Germany)

27 PCR DNA fragment extraction kit (Geneaid Biotech, Taiwan)

28 D-galactose (Sigma-Aldrich, USA)

29 Superoxide dismutase activity assay kit (Abcam, USA)

30 Catalase activity assay kit (Abcam, USA)

31 Glutathione peroxidase assay kit (Abcam, USA)

32 Lipid peroxidation (MDA) Assay Kit (Abcam, USA)

33 ELISA plate: 96- well plate: High binding (Corning, USA)

34 Recombinant rat TNF-α (R&D Systems, USA)

35 ELISA kit (R&D Systems, USA)

36 BioTek® Synergy™ HT (Multi-Detection Microplate Reader, USA)

37 Bovine serum albumin (BSA: Sigma, USA)

Methods

1 Selection of lactic acid bacteria

Lactic acid bacteria (LAB) were isolated from healthy human infant feces and were maintained as frozen (-80 °C) stocks (ethical approval number SWUEC37/2551). Fifty LAB isolates were randomly selected and grown on MRS (MRS; HiMedia Laboratories, India) agar at 37 °C for 24–48 h under anaerobic conditions (10% O_2; 10% CO_2, 80% N_2) and were sub-cultured three times before the experiment.

2 Preparation of lactic acid bacteria

2.1 Preparation of LAB viable cells and cell-free culture supernatants

Prior to use, Fifty LAB isolates were sub-cultured at least three times consecutively on MRS agar plate and incubated at 37°C for 48 h under anaerobic condition using anaerobic jar. Viable cells and cell-free culture supernatants (CFS) of LAB will be prepared as previously described with slight modification.[152] Briefly, LAB isolates were adjusted to approximately 109 CFU/ml in MRS broth. The preparation of viable cells, the cell pellets were collected and re-suspended in phosphate buffered saline (PBS; pH 7.2) after centrifuged at 4000 × g for 5 min at 4°C for use in the future study.

For preparation of CFS, the LAB isolates were re-suspended in MRS broth and incubated at 37 °C for 24–48 h under anaerobic conditions. The supernatants were collected after centrifuged at 4000 × g for 5 min at 4°C and filter sterilized through a 0.22 μm pore-size filter (Pall, USA). The resulting supernatant was used as the cell free supernatant. All LAB CFS were frozen at -20°C until analyses.

2.2 Preparation of LAB intracellular cell-free extracts

The intracellular cell-free extracts (ICFE) of LAB isolates were prepared as previously described.[153] Briefly, the cell pellets of LAB isolates were adjusted to approximately 10^9 CFU/ml in MRS broth and centrifuged at 4,000 *x g* for 10 min. Then, the cell pellets were washed twice with deionised water and resuspended in deionised water. After that, the cells pellets were incubated with 1 mg/ml lysozyme at 37°C for 30 min followed by ultrasonic disruption in five 1-min intervals in an ice bath (power 25-30%, Sonoplus HD 2070, Bandelin, Berlin, Germany). The cells were removed by centrifugation at 8,000 *x g* for 10 min at 4°C. The resulting supernatant were obtained as the intracellular cell-free extract of the LAB. All intracellular cell-free extracts were stored at -20°C until analyses.

3 Chemical assays for antioxidant activities of lactic acid bacteria

Antioxidant activities of probiotic isolates were evaluated using two different methods, 2,2-diphenyl-1-picryl-hydrazyl (DPPH), and hydroxyl radicals scavenging activity. Each method was repeated three times.

3.1 Determination of hydroxyl radical scavenging assay

The hydroxyl radical scavenging activity was measured based on the Fenton reaction as previously described by Wang et al. (2015)[153] with minor modifications. The Fenton reaction was freshly prepared by mixing 1.0 mL of 0.5 mM FeSO4, 0.5 mL of 0.435 mM brilliant green (Sigma® Aldrich, St. Louis, MO, USA) and 0.5 mL of samples as viable cells, CFS and ICFE, or deionized water used as a control. Addition of 0.75 mL of 3.0 % w/v H_2O_2 was then performed to initiate the reaction and incubated at room temperature for 20 min. The absorbance of the supernatant at 624 nm was measured. The brilliant green as a blank and 1% (w/v) ascorbic acid

was used as a positive control. The hydroxyl radicals scavenging activity was calculated as formula below:

$$\text{scavenging activity (\%)} = ((A_s - A_c) / (A_b - A_c)) \times 100.$$

Where A_s was the absorbance of samples, A_b was the absorbance of the blank control, and A_c was the same amount of water and sample mixture.

3.2 Determination of 2, 2-diphenyl-1-picrylhydrazyl radical scavenging

2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical is scavenged by antioxidants through the donation of a proton forming reduced DPPH. The free radical scavenging activity of the three samples (viable cell, CFS, ICFE) of LAB isolates were measured using a slight modification to the procedure previously reported.[152, 153] In brief, 0.2 mM solution of DPPH (DPPH, Sigma® Aldrich, St. Louis, MO, USA) in 95 % (v/v) ethanol was prepared. After 50 µL of samples solution were mixed with 100 µL of reagent DPPH solution into a 96 microculture plate. This microplate was vortexed and allowed to react for 30 min at room temperature in the dark. The supernatant was obtained by centrifugation at 4,000 × g at 25 °C for 10 min. The absorbance of the supernatant at 517 nm was measured. A positive control was 1% (w/v) ascorbic acid (Sigma® Aldrich, St. Louis, MO, USA). The percentage of scavenging activity was calculated according to the equation:

$$\text{scavenging activity (\%)} = (1 - (A_s - A_b) / A_c) \times 100$$

Where A_s was the absorbance of samples, A_b was the same amount of water and sample mixture, and A_c was the absorbance of the blank control.

4. Investigation of antioxidant activities of selected lactic acid bacteria on human colon carcinoma cells line

Lactic acid bacteria which showed significant scavenging activities in chemical assay were tested for antioxidant activities in cell lines to assess the ability of free radical reduction. The method was repeated three times.

4.1 Human colon carcinoma cells culture

Cell culture and *in vitro* bioassays were performed as previously described with some modifications.[41, 152, 153] Briefly, the human colon carcinoma (Caco-2) cell line were obtained from the American Type Culture Collection (ATCC HTB-37, Rockville, MD, USA) and were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco-Invitrogen, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco-Invitrogen, USA) and antibiotics (penicillin 100 U/mL and streptomycin 100 µg/mL) in a humidified atmosphere with 5% CO_2 at 37 °C. All cell culture experiments were carried out when the culture was approximately 60–80% confluent.

4.2 Measurement of intracellular free radical level

The intracellular ROS level was quantified by measuring the oxidation level of 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA; Sigma-Aldrich) as described by Guijie et al. (2018) with slight modifications. Briefly, Caco-2 cells suspension were seeded at a density of 2.4×10^4 cells/well on a black 96-well microplate in 100 µL/well and incubated at 37 °C for 24 h. After semi-confluent monolayers, the cells were treated with the samples of probiotic isolates for 24 h while control cells were exposed to deionized water. The cells were added with hydrogen peroxide (H_2O_2; Merck, Germany) to the final concentration of 100 µM for 2h to induce oxidative stress. Subsequently, the cells were stained by adding 25 µM DCFH-DA solution and incubated up to 30 min at 37°C after washing once with PBS to eliminate of the excess DCFH-DA. Fluorescence was measured at excitation and emission wavelengths of 485 and 535 nm. The cells of negative control wells were treated with DCFH-DA and H_2O_2 while the cells of blank wells were treated with DCFH-DA and PBS. The cellular antioxidant activity value was calculated from the area under the curve of fluorescence.

5 Measurement of general probiotic properties

Lactic acid bacteria which showed significant antioxidant activities were tested for acid tolerance, bile tolerance and adherence property with intestinal cell line in order to evaluate the relative survivability of selected probiotic LAB in the gastrointestinal tract.

5.1 Acid tolerance test

The survival of probiotic strain with antioxidant activity in acid conditions was investigated as described previously by Berne et al.[154] Briefly, MRS broth was adjusted with 1 N hydrochloric acid (HCL) to either pH 2.0, 3.0, and 4.0, respectively. MRS broth was used as negative control. The selected LAB was grown in MRS broth at 37 °C overnight, 0.1 mL aliquot containing 1×10^9 CFU/mL of selected LAB was inoculated into the modified MRS broth and incubated at 37°C for 3 h under anaerobic condition. The samples were left for 3 h at 37 °C. The viable cells were enumerated by spread plate on MRS agar incubated at 37 °C for 24-48 h. The viable cell counts were expressed as log colony forming units (log CFU/mL). All experiments were done in duplicate and two experiments were performed.

5.2 Bile tolerance test

The survival of probiotic strain with antioxidant activity in bile conditions was investigated as described previously by Berne et al. [154] Briefly, MRS broth was supplemented with bovine bile at 0.3% (w/v) or 0.8% (w/v) for bile salt conditions (Sigma, USA). The selected LAB was grown in MRS broth at 37 °C overnight, 0.1 mL aliquot containing 1×10^9 CFU/mL was inoculated in different acid and bile conditions. The samples were left for 3 h at 37 °C. The viable cells were prepared in PBS (pH 7.2) in 10-fold serial dilution and were enumerated by spread plate on MRS agar incubated at 37 °C for 24-48 h. The viable cell counts were expressed as log colony forming units (log CFU/mL). All experiments were done in duplicate and two experiments were performed.

5.3 *In vitro* adherence assay

Adherence was performed as previously described by Dimitrov et al. (2014)[155] with some modifications. A monolayer of Caco-2 cells were prepared as described in 4.1 The Caco-2 cells were seeded at a concentration of 1×10^5 cells/well in 24-well standard tissue culture plates (Falcon, USA) and cultured for 14 ± 1 days prior to use in adhesion assay. The cell culture was washed twice with PBS (pH 7.2) and added with serum-free and antibiotics-free medium at least 1 h before the adhesion assay.

Thereafter, 1 mL of probiotic strain suspension (10^9 CFU/mL in DMEM) was added to each well and incubated for 1 h at 37 °C in a humidified atmosphere containing 5% (v/v) CO_2. After incubation, the cells were washed thrice with a PBS solution to remove non-adherent bacteria, and lysed by addition of 0.5% (v/v) Triton® X-100 (Merck, Darmstadt, Germany) for 10 min. The lysed cells were plated on MRS agar and incubated at 37 °C for 48 h under anaerobic conditions. *L. rhamnosus* GG (LMG 18243) was used as a positive control. The adhesion percentage was then calculated as:

$$\% \text{ Adhesion} = (A_b / A_c) \times 100$$

Where A_b was the adhesion of viable bacteria cells, A_c was the total of bacteria added.

5.4 Hemolytic activity

Hemolytic activity of probiotic strain was determined by streaking on brain heart infusion agar (BHI; HiMedia, India) supplemented with 5% (v/v) defibrinated sheep blood. The overnight culture of the probiotic strain was streaked on sheep blood agar plates and incubated at 37 °C for 48 h under anaerobic conditions. The hemolytic reaction was recorded by observation. Probiotic strain showed no changes in the agar surrounding and under the colony indicated the absence of a hemolytic reaction (γ-hemolysis) were selected.

5.5 Antimicrobial activity

Antimicrobial activity of the probiotic strain against indicator strains was determined by agar-well diffusion method as described by Touré et al. (2003)[156] with slight modifications. Twelve indicator strains including *Staphylococcus aureus* ATCC 6538, methicillin resistant *S. aureus* DSMT 20654, *Streptococcus pneumoniae* DMST 4899, *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 25922, *Klebsiella pneumonia* DMST 8216, *Salmonella typhi* DMST 5784, *Shigella dysenteriae* DMST 15111, *Proteus mirabilis* DMST 8212, *Vibrio cholerae* DMST 2873, and *Pseudomonas aeruginosa* DMST 5870 were used. They were adjusted to the concentration of $1x10^8$ CFU/mL in PBS and were swabbed thrice on Mueller Hinton agar plates (MH; HiMedia

Laboratories, India) and 4 mm diameter well. Then, 25 µL of the naturalized and non-naturalized probiotic supernatant were filled in each well. MRS broth was used as a negative control. Following incubation at 37°C for 24 h, the diameters of inhibition zones were measured and recorded in millimeter (mm). The inhibition zone that less than 10 mm, 10 to 20 mm and more than 20 mm were considered as low (L), intermediate (M) and strong (S) inhibition, respectively. The experiment was performed twice, each in triplicate.

5.6 Antibiotic susceptibility testing

Antibiotic susceptibility of the probiotic strain was detected by the disc diffusion method according to Haghshenas et al. (2017)[157] with slight modifications. Briefly, the probiotic strain was adjusted to a concentration of 10^8 CFU/mL and swabbed on MH agar surfaces. Then, ten antibiotic discs including ampicillin (AMP;10 µg), chloramphenicol (CHL; 30 µg), erythromycin (ERY; 15 µg), gentamycin (GEN; 10 µg), nalidixic acid (NAL; 30 µg), penicillin G (PEN; 10 U), tetracycline (TET; 30 µg), vancomycin (VAN; 30 µg), bacitracin (B; 10 µg), streptomycin (S; 10 µg) (Bio-Rad Laboratories, Hercules, CA, USA) and a negative control filter paper containing autoclaved distilled water were placed on the surface of the agar. The plates were incubated for 24–48 h at 37 °C under anaerobic conditions. After the incubation, the diameter of the inhibition zone was measured and the results were expressed in terms of resistance according to Charteris WP et al. (1998).[158]

6 Genotypic identification of selected probiotic lactic acid bacteria

The selected probiotic LAB isolates were analyzed for 16S ribosomal RNA gene sequence as described previously.[159] The 16S rRNA sequence coding region were amplified by PCR. The colony of selected probiotic LAB were resuspended in 300 µl of milliQ water and added 100 ul of PCR Master Mix (5 U Taq DNA polymerase, 2 mM dNTP, 25 mM MgCl2, 10X Taq Buffer, deionized H_2O). The 16S rRNA gene sequences were using universal primers 20F (5'-AGTTTGATCCTGGCTC-3') and 1500R (5'-AAGGAGGTG ATCCAGCC-3'). Amplification mixtures were subjected to 3 min of denaturation at 94°C; 30 cycles of denaturation at 94°C for 20 s, annealing at 50°C for

1 min 20 sec, and extension at 72°C for 2 min; followed by a final extension period of 3 min at 72°C. PCR products was purified using a QIA quick PCR Purification Kit (QIAGEN, USA) and analyzed sequence by U2Bio in Korea. The nucleotide sequences were then identified and aligned with published sequences of the type strains from the National Center for Biotechnology Information (NCBI) GenBank database and EzTaxon bioinformatics software. The percent identities of the bacterial isolates were determined on the basis of highest score. The closest relatives of the 16S rRNA gene sequences were evaluated. A similarity of ≥ 99% to 16S rRNA gene sequences of type isolates were used as the criterion for identification.

Phylogenetic tree was constructed by the neighbor-joining method[160] using MEGA version 7.0.[161] Bootstrap resampling analysis of 1000 replicates was performed to estimate the confidence levels of the tree topologies.[162]

7 Assessment of antioxidant effects of the selected probiotic lactic acid bacteria on D-galactose induced rats

Selected probiotics lactic acid bacteria which showed the best antioxidant activities in chemical assay and cell lines, was assessed for antioxidant effect on D-galactose induced rats.

7.1 Animals and administration

Eight-week-old male Sprague Dawley rats with body weights of 260 - 280 g were purchased from the Nomura Siam international Co., Ltd. The animals were maintained on a 12 h light / 12 h dark cycle at a constant temperature (22±1°C) and were allowed free access to food and water during the experimental period.

After one week acclimatization to the laboratory environment, rats were randomly divided into 4 groups of 5 rats in each group.[42, 125, 153] The normal control group (1st group) was administered by oral gavage with normal saline at a dose of 20 ml/kg body weight once daily. The D-galactose (D-gal model) group (2nd group) was treated with normal saline and subcutaneously injected with 5% (w/v) D-gal dissolved in normal saline at a dose of 500 mg/kg body weight once daily for 8 weeks. The probiotic group (3rd group) was received the best of probiotic LAB at a concentration of

10^9 CFU/day and injected with 5% (w/v) D-gal. The last group (4^{th} group) was treated with ascorbic acid (1.0 mg/ml) at the same dose and frequency, and injected with 5% (w/v) D-gal.

Twenty-four hours after the final administration, the rats of all groups were weighed. After that, blood samples of each group were collected under an aesthesia by cardiac puncture in vials containing heparin and sodium chloride. Serum samples were obtained by centrifugation at 3,000 *x g* for 10 min at 4°C and stored at -80°C for antioxidant activity analysis and were examined the level of glucose, lipid profile and liver function test by Professional laboratory, Thailand (PROLAB). The liver, spleen, stomach and total fat were removed and weighed. The liver was homogenized in ice-cold normal saline to prepare 10% (w/v) homogenate. The homogenates were then centrifuged at 3,000 *x g* for 10 min at 4°C and the protein concentration were determined.

7.2 Determination of antioxidant activities in serum and liver tissues

Superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), catalase and malondialdehyde (MDA) levels in serum were determined with commercially available assay kits according to the manufacturer's instructions (Abcam, USA).[42, 125]

7.2.1 Measurement of superoxide dismutase activity

For superoxide dismutase (SOD) assay, each well of plate was filled with 200 µl of WST working solution and 20 µl of sample. After addition of 20 µl of enzyme working solution to initiate the reaction and the mixture was incubated at 37°C for 20 min. Absorbance was read at 450 nm with a microplate reader. Results of SOD concentration was quantified from standard curve and expressed as U/ml.

7.2.2 Measurement of glutathione peroxidase activity

For glutathione peroxidase (GSH-Px) assay, briefly, a 2-50 microliter of sample was diluted with assay buffer and adjusted volume to 50 µL/well. After that, 40 microliter of reaction mix contained 33 µl of assay buffer, 3 µl of NADPH solution (40 mM), 2 µl of GR solution and 2 µl of GSH solution was mixed vigorously and

incubated at room temperature for 15 min to deplete all GSSG in the samples. After addition of 10 µl cumene hydroperoxide solution to start the glutathione peroxidase (GSH-Px) reaction and change in absorbance per 5 min were recorded at max of 340 nm. The enzyme activity was quantified from standard curve and expressed as U/ml.

7.2.3 Measurement of catalase activity

For catalase assay, samples were diluted then final volume at 78 µl/well with catalase assay buffer. After addition of 12 µl of fresh 1 mM H_2O_2 solution and incubated at 25°C for 30 min. Later, a 10 microliter of stop solution was added to each sample and mixed with 50 µl of developer mix contained 46 µl of catalase assay buffer, 2 µl of OxiRed Probe and 2 µl of HRP solution. After incubated at 25°C in the dark for 10 min, the absorbance of the mixture at 570 nm was measured immediately with a spectrophotometer. The enzyme activity was quantified from standard curve and expressed as U/ml.

7.2.4 Measurement of lipid peroxidation

The lipid peroxidation was determined by the TBA reaction with malondialdehyde (MDA), a product formed due to peroxidation of lipids. Generation of MDA-TBA, a 200 microliter of sample was mixed with 600 µl of TBA reagent and incubated at 95°C for 60 min. After cooling to room temperature in an ice bath for 10 min, 300 µl of n-butanol was added to the precipitate MDA-TBA. Top layer containing n-butanol was transferred to a new tube by centrifugation at 16,000 *x g* for 3 min at RT. Then, n-butanol was evaporated by incubating sample in a dry-oven at 55°C. The MDA-TBA was dissolved in 200 µl sterile deionized water and then transferred into the 96-well plate microplate for analysis by measurement at 532 nm using a microplate reader. Concentration of MDA in the samples were quantified from standard curve and expressed as nmol/ml.

7.3 Measurement of tumor necrosis factor-alpha level in liver tissues

Tumor necrosis factor -alpha (TNF-α) level in homogenized liver tissues was measured with cytokine-specific sandwich quantitative enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (R&D

Systems, USA).[163, 164] Briefly,100 µl mouse anti-rat TNF-α antibodies as capture antibodies was coated on 96 ELISA microplates and incubated at 4°C overnight. The plate was removed excess capture antibody by washed three times with PBS containing 0.05% Tween 20 (PBST) and reduced non-specific binding by added 300 µl of 1% Bovine serum albumin (BSA: Sigma, USA) for 2 h. After that, adding of 100 µl of Standard recombinant rat TNF-α or homogenized liver tissues were incubated overnight. After washing three times with PBST, 100 µl of biotinylated goat anti-rat TNF-α antibodies was added as detection antibodies. After incubated for 2 h, the plates here was incubated with 100 µl of streptavidin-horseradish peroxidase conjugate at room temperature for 20 min. After that, 100 µl/well solution of tetramethyl benzidine substrate (TMB) was added and incubated for 20 min as color indicator by bound peroxidase conjugate. The plates were read at 450 nm using a BioTek® Synergy™ HT (Multi-Detection Microplate Reader, USA). The TNF-α levels are expressed in pg/ml by calculating the quantity from the standard curve.

7.4 Analysis of intestinal microbiota

Stool samples were collected pre and post intervention for intestinal microbiota analysis by plate count.[165] Briefly, one gram of fresh samples were homogenized with 9 ml of PBS (pH 7.2) and were prepared with PBS in 10 fold serial dilution. The viable cells count of bacteria were enumerated by spread plate on eosin methylene blue (EMB) agar, MRS agar and *Bifidobacterium* selective medium (BSM) agar supplemented with BSM supplement (Sigma-Aldrich, USA). The viable cells were enumerated by spread plate on MRS agar incubated at 37 °C for 24-48 h under anaerobic condition. The viable cell counts were expressed as log colony forming units (log CFU/mL). All experiments were done in duplicate and two experiments were performed.

8 Measurement for chemical and physical properties of milk fermented with selected probiotic lactic acid bacteria

8.1 Optimization of yoghurt with selected probiotic strain

Yoghurt starter culture YoFlex–YC-X11 containing *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* was obtained from CHR HANSEN's Lab., Inc. (Milwaukee, WI). Yoghurt starter culture was isolated and identified according their characteristics. Pasteurized milk was kindly provided by CP-meiji (CP-meiji co. Ltd., Thailand), and was heated at 85 ˚C for 30 min, and left in the water bath until its temperature was equal to 40-43 ˚C. The selected probiotic strain, *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* were each inoculated at 10^9 CFU/mL. Yoghurt made with starter culture alone was used as a control. Fermentation was conducted at 43 ˚C until pH 4.60. The fermented milk samples were analyzed weekly during 4 weeks of storage at 4 ± 1 ˚C. The viability of microorganisms, physicochemical properties including pH changes, texture, syneresis, and rheological properties were determined.

8.2 Measurement of viability

Yoghurt was prepared (10^{-1}-10^{-5}) 10-fold serial dilutions of each sample. Viable bacterial cells were determined by spreading each dilution onto MRS agar plates. The plates were incubated at 37°C for 48 h under anaerobic condition generated using an anaerobic jar. Viable bacteria were counted and displayed as cell numbers and the log of colonies grown on MRS agar.

8.3 Measurement of pH

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

8.4 Measurement of texture

Yoghurt samples formed in a glass container (52 mm in diameter until a height of 50 mm) were analyzed for firmness using a compression test carried out with a TA.XT plus Texture Analyzer (Stable Micro Systems, Surrey, UK) equipped with a 20 mm acrylic cylinder probe. 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.

8.5 Measurement of syneresis

The syneresis of both yogurt were measured according to the method proposed by Vivar-Quintana et al. (2006)[166] with slight modifications. Briefly, 30 g yoghurts formed in centrifuge tubes were centrifuged at 680 × g using Sorvall Legend T plus Centrifuge (Thermo Scientific, USA) at 4°C for 10 min. The weight percentage of liquid released after centrifugation was calculated according to the equation:

$$\% \text{ Syneresis} = \frac{\text{Weight of whey (g)}}{\text{Total weight of milk (g)}} \times 100$$

8.6 Measurement of rheological properties

Dynamic oscillatory measurements were performed using a controlled stress rheometer (HAAKE Mars 40, Thermo Fisher Scientific, Karlsruhe, Germany) as described by Sah et al. (2016)[167] with minor modifications. The rheometer was equipped with a plate and cone geometry (60 mm diameter, 1 mm gap) and controlled the temperature at 4°C. The yoghurt sample homogeneously stirred was pre-sheared for 30 s at a shear rate of 500 s^{-1}, and structural recovery by equilibration for 300 s. The test frequency was range from 0.1 to 10 Hz, and the strain amplitude was 0.5 %. The storage modulus (G´) and loss modulus (G") were reported at a frequency of 1 Hz.

The apparent viscosity of yoghurt samples was performed after equilibration for 300 s in the same sample. The shear rate logarithmically added from 0.01 to 100 s^{-1}. The apparent viscosity was reported at 20 s^{-1}.[168]

9. Statistical analysis

All experiments, data of all experiments were assessed using ANOVA and followed by post hoc comparisons using the Tukey's test for percentage of hydroxyl radical scavenging, DPPH radical scavenging, intracellular free radical level, SOD level, GSH-Px level, catalase level, MDA level, and TNF-α level. The result of acid-bile tolerance, adhesion ability, viability of fermented milk, and physiochemical of fermented milk were used Student's t-test. Statistical significance of differences were evaluated by GraphPad Prism version 5.01. Significance of differences will be considered significant at $P < 0.05$.

CHAPTER IV
RESULTS

1 Selection of lactic acid bacteria

Fifty isolates of healthy infant feces (SWUEC 37/2551) were randomly selected from frozen stocks (-80°C). Each isolate was presumptively identified by Gram-staining for morphological detection and catalase examination. Moreover, the isolates were grown under facultative aerobic conditions or anaerobic conditions. Isolates of catalase-negative, gram-positive, bacilli, short rods, coccobacilli and regular rods were selected and maintained in frozen stock (-80ºC) for used in this study.

Table 4 50 selected lactic acid bacteria isolates.

Isolates no.	Colony morphology	Gram morphology
MSMC5-1	Medium colonies, yellow, convex	Gram positive long bacilli, big rod
MSMC6-5	Medium colonies, yellow, circular, flat	Gram positive long bacilli, big rod
MSMC7-1	Medium colonies, yellow, circular	Gram positive coccobacilli
MSMC12-2	Small colonies, white, circular	Gram positive medium bacilli
MSMC20-1	Large colonies, white, circular	Gram positive medium bacilli, slender
MSMC25-2	Medium colonies, yellow, circular, flat	Gram positive medium bacilli, pinpoint
MSMC28-2	Large colonies, mucoid, white, circular	Gram positive medium bacilli
MSMC35-4	Medium colonies, white, circular	Gram positive medium bacilli
MSMC36-9	Large colonies, white, convex	Gram positive medium bacilli
MSMC37-3	Large colonies, white, convex	Gram positive long bacilli, big
MSMC37-4	Large colonies, white, convex	Gram positive very long bacilli

Table 4 (continued)

Isolates no.	Colony morphology	Gram morphology
MSMC37-6	Large colonies, yellow, flat	Gram positive medium bacilli
MSMC39-1	Large colonies, white, convex	Gram positive medium bacilli
MSMC39-3	Large colonies, white, convex	Gram positive long bacilli, granule
MSMC39-5	Large colonies, yellow, circular	Gram positive medium bacilli
MSMC40-2	Large colonies, white, convex	Gram positive medium bacilli
MSMC40-7	Medium colonies, white, convex	Gram positive short bacilli
MSMC 42-1	Small colonies, white, convex	Gram positive short bacilli, slender
MSMC56-1	Medium colonies, yellow, convex	Gram positive short bacilli
MSMC57-1	Small colonies, white, shiny	Gram positive short bacilli, slender
MSMC57-2	Medium colonies, yellow, shiny	Gram positive short bacilli, slender
MSMC63-2	Small colonies, white, convex	Gram positive coccobacilli
MSMC64-1	Medium colonies, yellow, flat	Gram positive medium bacilli, pinpoint
MSMC81-2	Medium colonies, white, convex	Gram positive short bacilli, curve
MSMC83	Small colonies, white, convex	Gram positive short bacilli, dumbbell
MSMC95-4	Medium colonies, yellow, flat	Gram positive coccobacilli
MSMC96-2	Small colonies, white, convex	Gram positive short bacilli, curve
MSMC99-1	Medium colonies, yellow, convex	Gram positive short bacilli, curve
MSMC104-2	Large colonies, yellow, circular	Gram positive coccobacilli
MSMC105-1	Large colonies, white, convex	Gram positive medium bacilli
MSMC105-3	Large colonies, white, shiny	Gram positive medium bacilli
MSMC111-1	Large colonies, white, circular	Gram positive medium bacilli, slender
MSMC111-2	Medium colonies, white, circular	Gram positive coccobacilli
MSMC112-2	Large colonies, yellow, opaque	Gram positive long bacilli, big rod

Table 4 (continued)

Isolates no.	Colony morphology	Gram morphology
MSMC120-2	Medium colonies, white, circular, convex	Gram positive short bacilli, granule
MSMC171-1	Medium colonies, white, shiny	Gram positive medium bacilli
MSMC177-1	Very small colonies, white, circular	Gram positive short bacilli
MSMC203-3	Small colonies, white, convex	Gram positive medium bacilli, granule
MSMC211-1	Large colonies, yellow, convex	Gram positive coccobacilli
MSMC239-1	Large colonies, white, convex	Gram positive coccobacilli
MSMC248-1	Small colonies, yellow, convex	Gram positive coccobacilli
MSMC258-1	Small colonies, yellow, convex	Gram positive coccobacilli
MSMC261-2	Very small colonies, white, convex	Gram positive coccobacilli
MSMC280-1	Small colonies, white, convex	Gram positive short bacilli
MSMC281-1	Small colonies, white, convex	Gram positive short bacilli, big
MSMC290-1	Medium colonies, yellow, flat	Gram positive short bacilli
MSMC296-1	Medium colonies, yellow, convex	Gram positive short bacilli, slender
MSMC300-2	Small colonies, white, convex	Gram positive very short bacilli
MSMC302-1	Medium colonies, yellow, flat	Gram positive very short bacilli

2 Antioxidant activities of lactic acid bacteria isolates

2.1 Antioxidant activity by hydroxyl radical scavenging activity

Among 50 LAB isolates, four LAB isolates including MSMC36-9, MSMC37-3, MSMC37-4 and MSMC83, showed significant hydroxyl free radical scavenging activity (Table 5). The hydroxyl free radicals scavenging effects of LAB isolates were range 10.01% to 18.57% for viable cells, 13.99% to 23.39% for cell free supernatants and 33.98% to 43.87% for intracellular cell free extracts. Intact cells of LAB isolates had

higher hydroxyl radical activity as compared to CFS. Interestedly, the hydroxyl radicals scavenging activity of ICFE exhibited the highest scavenging capability. The viable cells of MSMC83 exhibited significant the higher hydroxyl free radical scavenging activity than other LAB isolates ($P < 0.05$) as shown in Figure 6. In CFS of MSMC83 and MSMC36-9 exposed the highest hydroxyl maximum (Figure 7). Among the four LAB isolates, the ICFE of MSMC83 showed significant the highest hydroxyl radicals scavenging activity ($P < 0.05$) as shown in Figure 8.

Table 5 Hydroxyl free radical scavenging activity of 4 selected LAB isolates.

Sample		Hydroxyl free radical scavenging activity (%)
MSMC36-9	Viable cells	12.67 ± 1.38
	Cell free supernatant	22.57 ± 1.96
	Intracellular cell free extract	40.43 ± 0.90
MSMC37-3	Viable cells	10.01 ± 1.77
	Cell free supernatant	15.11 ± 1.66
	Intracellular cell free extract	35.34 ± 1.23
MSMC37-4	Viable cells	9.12 ± 1.17
	Cell free supernatant	13.99 ± 1.55
	Intracellular cell free extract	33.98 ± 1.14
MSMC83	Viable cells	18.57 ± 1.21
	Cell free supernatant	23.39 ± 1.83
	Intracellular cell free extract	43.87 ± 1.34
Ascorbic acid		23.39 ± 0.23
PBS		3.99 ± 0.23
MRS broth		3.79 ± 0.43

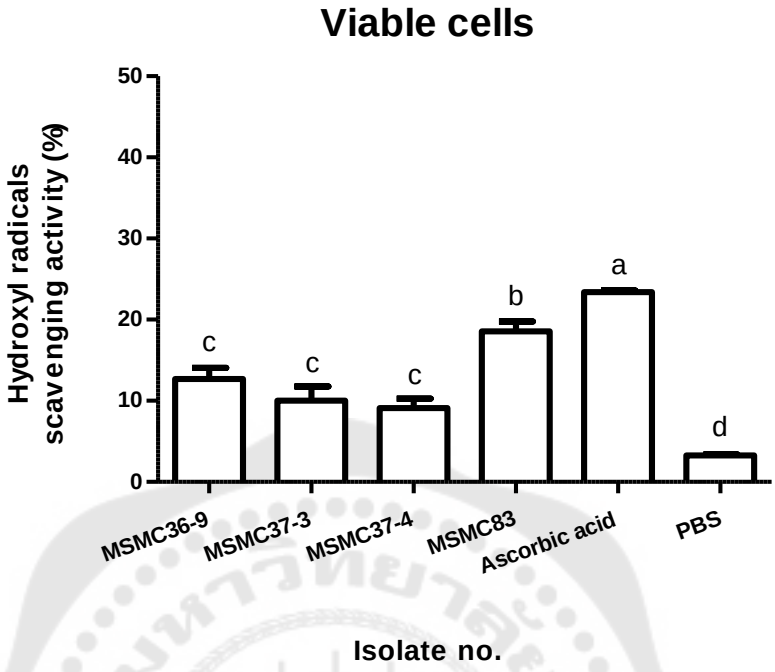

Figure 6 Antioxidant activity of viable cells of LAB isolates in hydroxyl radicals scavenging.

Values are expressed as mean and SD (n = 3). The different superscript letters a-d in the LAB isolates represent statistical significance ($P < 0.05$).

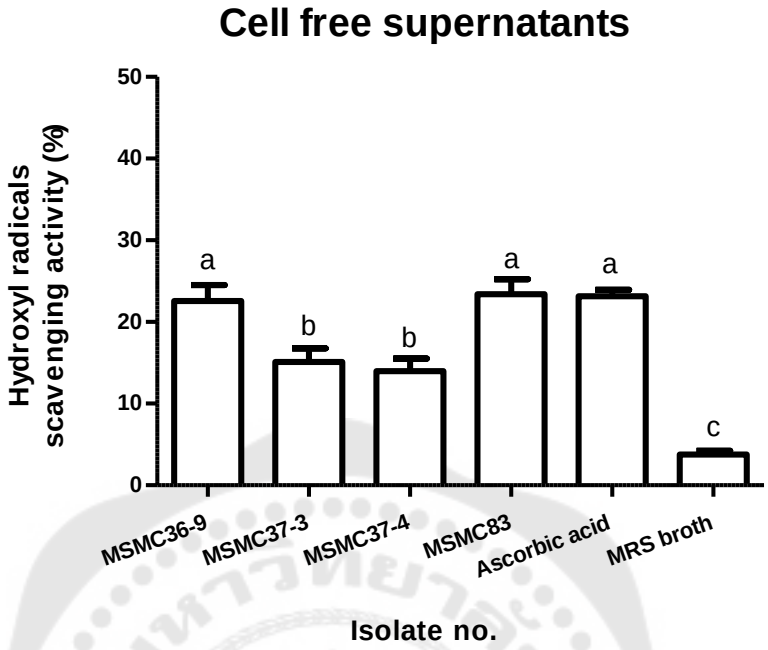

Figure 7 Antioxidant activity of CFS of LAB isolates in hydroxyl radicals scavenging assays.

Values are expressed as mean and SD (n = 3). The different superscript letters a-c in the LAB isolates represent statistical significance ($P < 0.05$).

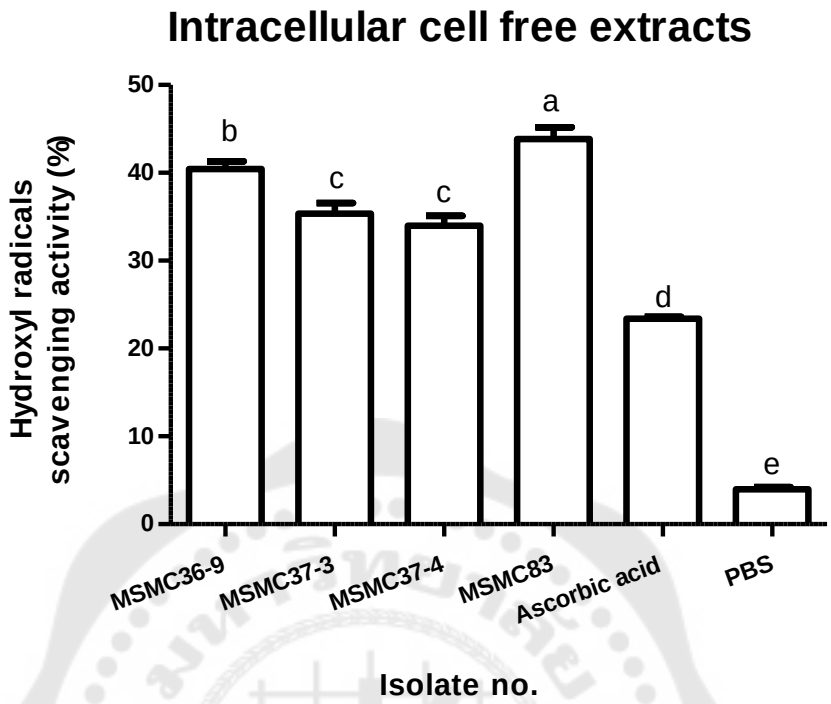

Figure 8 Antioxidant activity of ICFE of LAB isolates in hydroxyl radicals scavenging assays.

Values are expressed as mean and SD (n = 3). The different superscript letters a-c in the LAB isolates represent statistical significance ($P < 0.05$).

2.2 2, 2-diphenyl-1-picrylhydrazyl radical scavenging

The DPPH radical scavenging activity was used as a tool to investigate the antioxidative activity of the 50 probiotic isolates. Four LAB isolates including MSMC 36-9, MSMC37-3, MSMC37-4 and MSMC83, was found to have antioxidant property. The results of the radical scavenging methods of the viable cells, CFS and ICFE of 4 LAB isolates exhibited as shown in table 6. When the viable cells were measured, the DPPH radical scavenging activity of 4 LAB isolates ranged from 12.99% to 18.31% (Figure 9). Compared to viable cells, the CFS showed scavenging activity with values ranging from 18.17 % to 38.21% (Figure 10). The ICFE was significant the highest DPPH radical scavenging activity (Figure 11). The CFS and ICFE of MSMC83 isolate were found to have maximum DPPH radical scavenging activity compared to other isolates. There, 4 LAB isolates were found to have higher antioxidant potential and was selected for further cell antioxidant assays.

Table 6 DPPH radical scavenging activity of 4 selected LAB isolates.

Sample		DPPH radical scavenging activity (%)
MSMC36-9	Viable cells	18.31 ± 2.19
	Cell free supernatant	29.34 ± 1.43
	Intracellular cell free extract	45.54 ± 2.50
MSMC37-3	Viable cells	13.57 ± 1.90
	Cell free supernatant	21.74 ± 2.82
	Intracellular cell free extract	30.73 ± 2.89
MSMC37-4	Viable cells	13.00 ± 1.80
	Cell free supernatant	18.18 ± 1.54
	Intracellular cell free extract	25.45 ± 2.79
MSMC83	Viable cells	17.32 ± 1.18
	Cell free supernatant	38.2 ± 1.16
	Intracellular cell free extract	59.41 ± 1.47
Ascorbic acid		82.35 ± 0.45
PBS		1.78 ± 0.29
MRS broth		1.65 ± 0.14

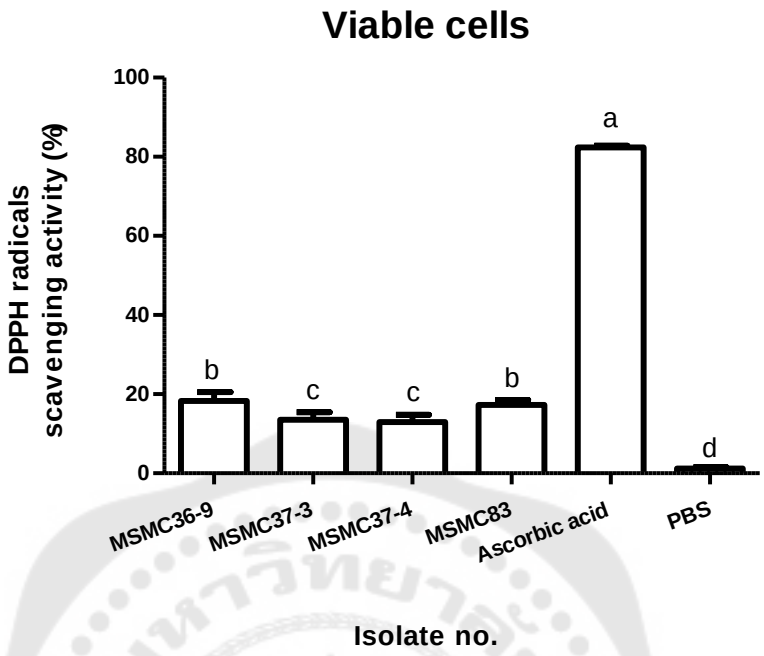

Figure 9 Antioxidant activity of viable cells of LAB isolates in DPPH radicals scavenging assays.

Values are expressed as mean and SD (n= 3). The different superscript letters a-d in the LAB isolates represent statistical significance ($P < 0.05$).

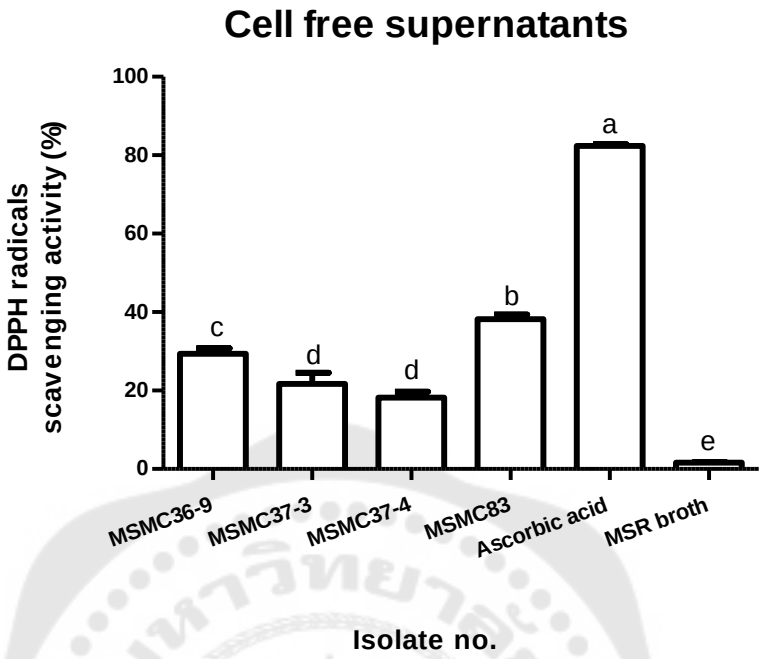

Figure 10 Antioxidant activity of CFS of LAB isolates in DPPH radicals scavenging assays.

Values are expressed as mean and SD (n = 3). The different superscript letters a-e in the LAB isolates represent statistical significance ($P < 0.05$).

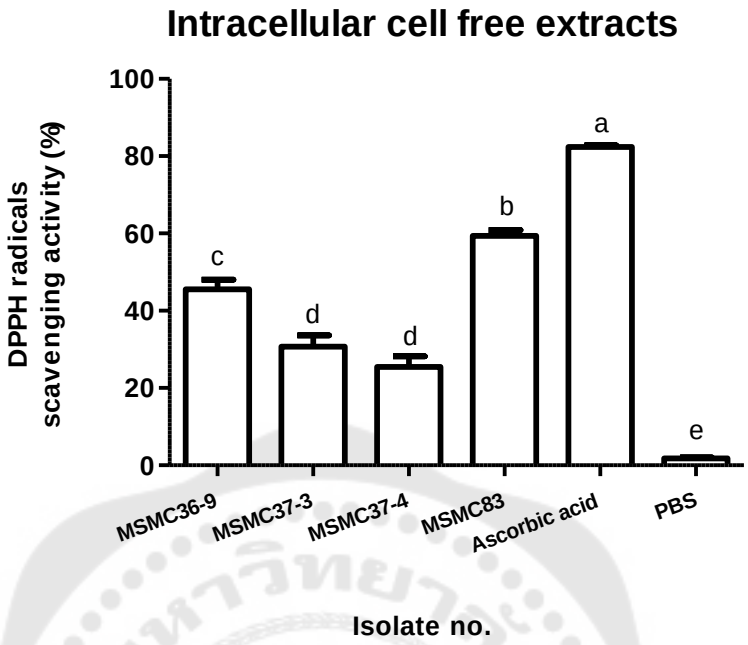

Figure 11 Antioxidant activity of ICFE of LAB isolates in DPPH radicals scavenging assays.

Values are expressed as mean and SD (n = 3). The different superscript letters a-e in the LAB isolates represent statistical significance ($P < 0.05$).

3 antioxidant activities of selected LAB on human colon carcinoma cells line

3.1 Cell Cytotoxicity

The cytotoxic effects of Caco-2 cells line were determined in viable cells, CFS and ICFE of selected LAB using the trypan blue exclusion assay. It was found that the viable cells, CFS and ICFE exhibited toxicity on Caco-2 cells as similar to control (Figure 12). Negative control was untreated cells (mean ± SD, n = 3).

Figure 12 Cytotoxicity of the viable cells, CFS, and ICFE of selected LAB on human colon carcinoma cells line.

3.2 Quantification of cellular oxidative stress by 2',7'- dichlorofluorescein diacetate assay

The intracellular ROS analysis was applied in the present study to quantitatively evaluate the antioxidant activity of viable cells, CFS and ICFE of LAB isolates in Caco-2 cells as shown in Figure 13-15. All selected LAB inhibited free radical in Caco-2 cells. In table 7, the ICFE showed significantly higher antioxidant activity than the viable cells and CFS ($P < 0.05$). Viable cells and CFS of MSMC83 showed higher cellular antioxidant activity values than the other selected LAB ($P < 0.05$). The ICFE of MSMC83 and MSMC36-9 showing the maximum antioxidant activity of approximately 73.24% and 80.23%, respectively.

Table 7 Cellular antioxidant activity values of 4 selected LAB isolates.

Sample		Cellular antioxidant activity values (%)
MSMC36-9	Viable cells	32.27 ± 2.96
	Cell free supernatant	57.21 ± 2.47
	Intracellular cell free extract	73.24 ± 4.85
MSMC37-3	Viable cells	22.56 ± 1.39
	Cell free supernatant	44.90 ± 2.28
	Intracellular cell free extract	51.23 ± 3.82
MSMC37-4	Viable cells	22.42 ± 1.74
	Cell free supernatant	39.79 ± 1.10
	Intracellular cell free extract	50.91 ± 1.29
MSMC83	Viable cells	38.00 ± 1.02
	Cell free supernatant	63.2 ± 1.31
	Intracellular cell free extract	80.21 ± 0.98
Ascorbic acid		73.55 ± 0.81
PBS		3.78 ± 1.98
MRS broth		6.98 ± 1.47

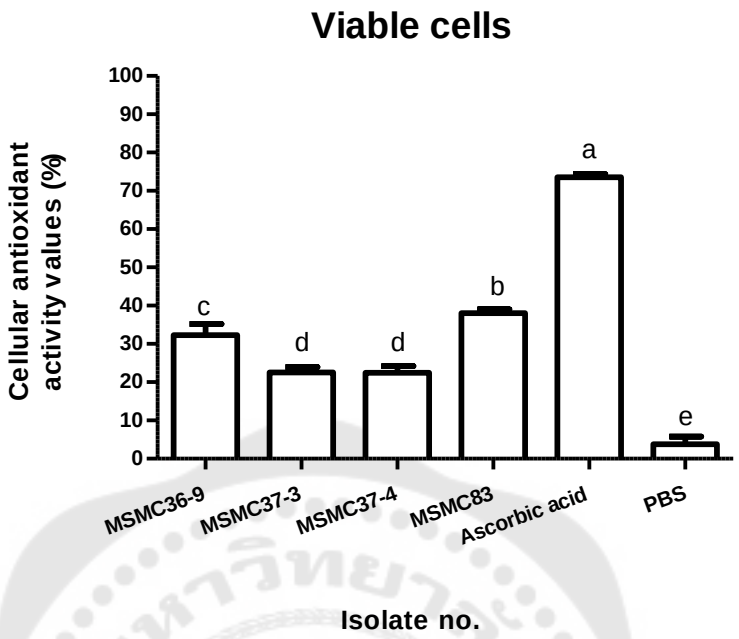

Figure 13 Cellular antioxidant activity of viable cells of LAB isolates in Caco-2 cells.

Values are expressed as mean and SD (n = 3). The different superscript letters a-e in the LAB isolates represent statistical significance ($P < 0.05$).

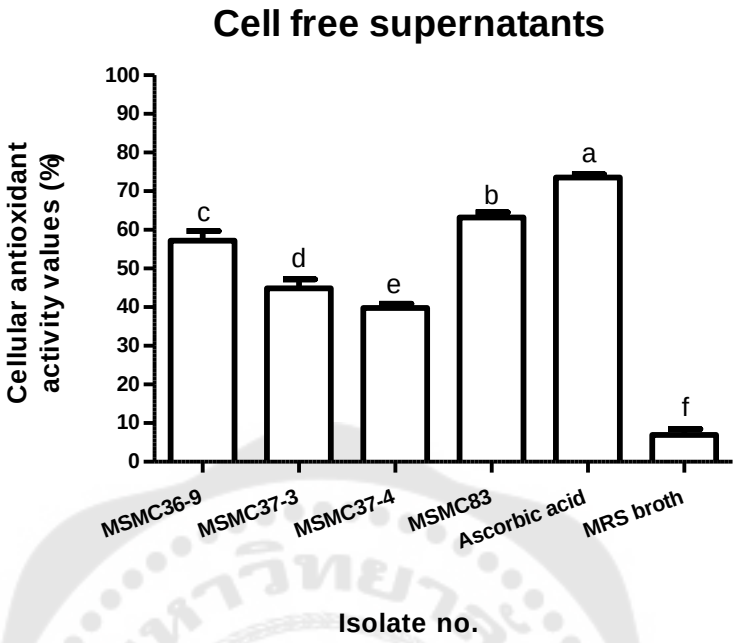

Figure 14 Cellular antioxidant activity of CFS of LAB isolates in Caco-2 cells.

Values are expressed as mean and SD (n = 3). The different superscript letters a-f in the LAB isolates represent statistical significance ($P < 0.05$).

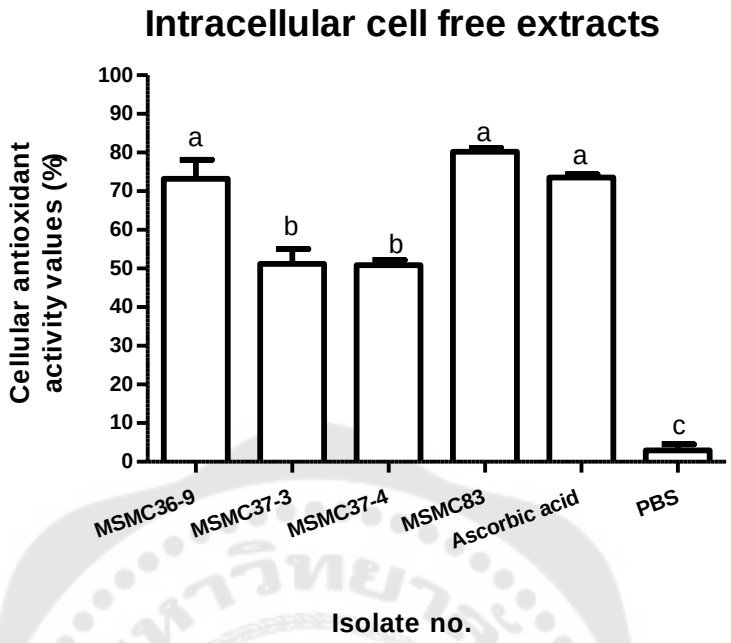

Figure 15 Cellular antioxidant activity of ICFE of LAB isolates in Caco-2 cells.

Values are expressed as mean and SD (n = 3). The different superscript letters a-c in the LAB isolates represent statistical significance ($P < 0.05$).

4 General probiotic properties

Four lactic acid bacteria isolates which showed significant antioxidant activities were evaluated for acid tolerance, bile tolerance and adherence property with intestinal cell line in order to determine the relative survivability of selected probiotic LAB in the gastrointestinal tract.

4.1 Acid tolerance test

One of the important criteria for selection of probiotics is the tolerance to low pH condition in the stomach which allows them to survive on their way through the gastrointestinal tract. The LAB with the antioxidant activity, MSMC36-9, MSMC37-3, MSMC37-4 and MSMC83 were selected for acid tolerance test (Figure 16-19, Table 8). It retained varying survival rate in MRS broth at pH 2.0, 3.0 and 4.0 when compared to MRS control after 3 h of incubation. The number of MSMC83 exposure at pH 2.0 was

6.785 Log CFU/mL. Whereas the MSMC36-9, MSMC37-3 and MSMC37-4 did not survive at pH 2.0. The survival rate of all isolates at pH 3.0 was 95.94 – 99.55 %, while at pH 4.0 the survival rate increased to 97.74 - 102.5 %. Therefore, all selected LAB were able to rate tolerate found in acidic conditions at pH 3.0 and 4.0 when isolate compared to MRS control. The highest survival was for MSMC83 isolate while the least survival was observed for MSMC37-3 isolate.

Table 8 The survival of selected LAB after incubation in different pH values.

Isolate no.	Number of viable cells (Log CFU/mL)			
	MRS 3 hr (control)	pH 2.0	pH 3.0	pH 4.0
MSMC36-9	8.658 ± 0.08	0.00***	8.401 ± 0.06*	8.789 ± 0.05^{NS}
MSMC37-3	9.053 ± 0.04	0.00***	8.633 ± 0.18*	9.232 ± 0.07^{NS}
MSMC37-4	9.006 ± 0.02	0.00***	8.966 ± 0.02**	9.232 ± 0.06^{NS}
MSMC83	9.326 ± 0.04	6.785 ± 0.03***	8.854 ± 0.15**	9.116 ± 0.01^{NS}

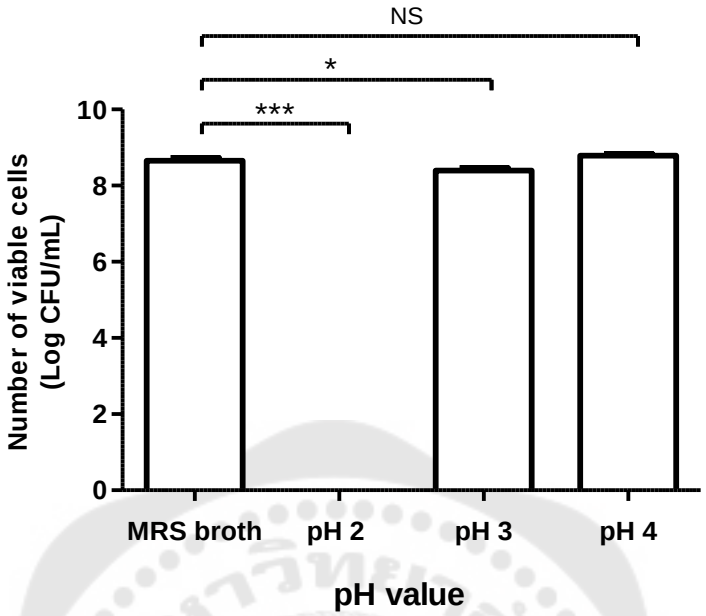

Figure 16 Acid tolerance of MSMC36-9.

Statistical analysis: student's *t*-test with one-tail distribution. Significantly different compared to control 3 hr (* $P < 0.05$; *** $P < 0.001$).

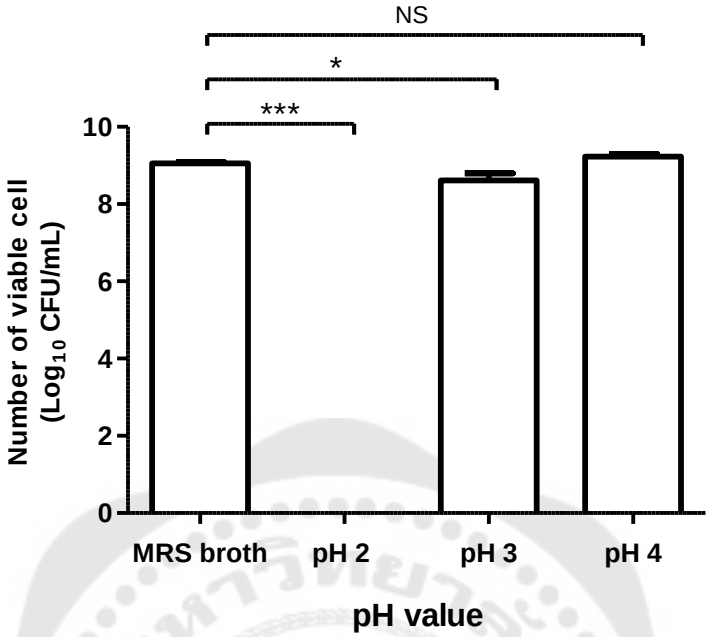

Figure 17 Acid tolerance of MSMC37-3.

Statistical analysis: student's *t*-test with one-tail distribution. Significantly different compared to control 3 hr. (* $P < 0.05$; *** $P < 0.001$).

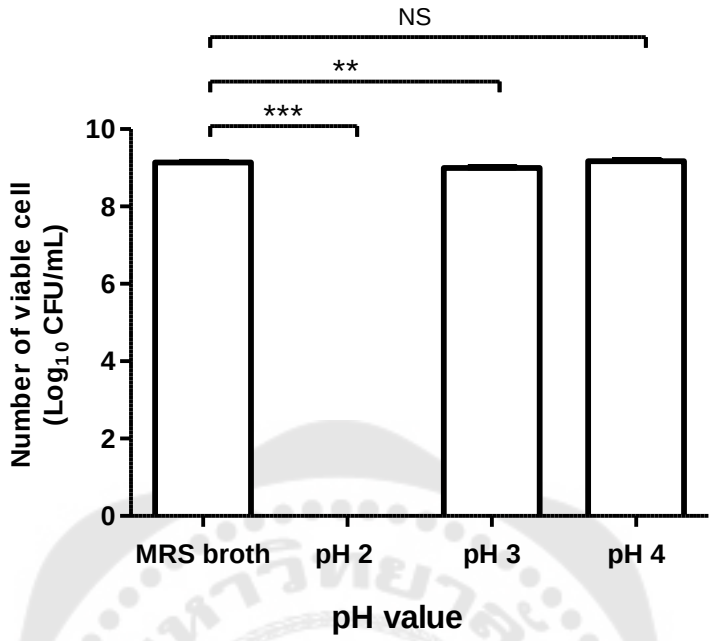

Figure 18 Acid tolerance of MSMC37-4.

Statistical analysis: student's *t*-test with one-tail distribution. Significantly different compared to control 3 hr (** $P < 0.01$; *** $P < 0.001$).

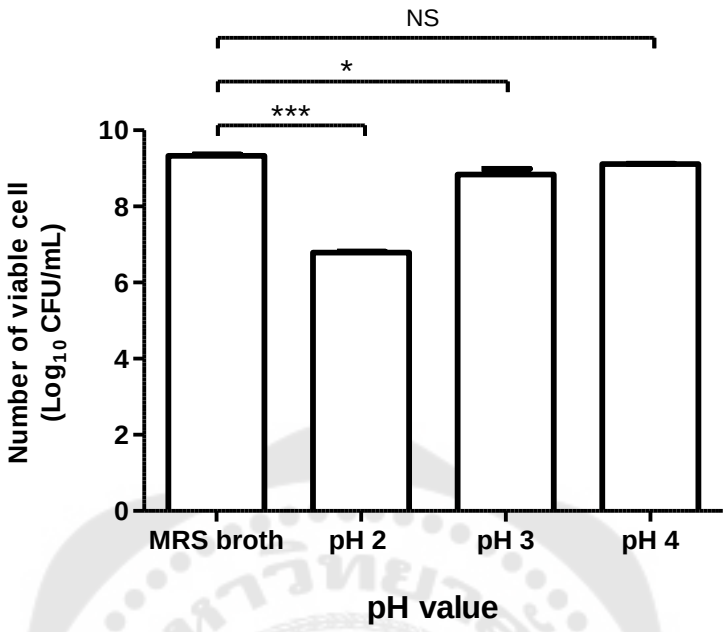

Figure 19 Acid tolerance of MSMC83.

Significantly different compared to control 3 hr (* $P < 0.05$; *** $P < 0.001$).

4.2 Bile tolerance test

MSMC36-9, MSMC37-3, MSMC37-4 and MSMC83, were screened for their ability to tolerate bile salt. All selected LAB isolates displayed bile tolerance with varying level at 0.3% and 0.5% bile salt concentrations after incubation for 3 hr. MSMC37-3, MSMC37-4 and MSMC83 were able to grow at 0.26%, 1.41%, and 1.07%, respectively while the viability of MSMC36-9 isolate was decreased 0.152% at 0.3% of bile salt as compared to MRS control. All selected LAB isolates survived well under 0.8 % bile conditions, and the viable cell count ranged from 8.065 ± 0.042 to 9.002 ± 0.015 log CFU/mL. All isolates showed no significant differences after incubation in both bile salt conditions, compared with MRS control (Figure 20-23, Table 9). Therefore, all isolates tolerated to 0.3 % and 0.8 % bile salt condition.

Table 9 The survival of selected LAB after incubation in different bile salt.

Isolate no.	Number of viable cells (Log CFU/mL)		
	MRS (control)	0.3% bile salt	0.8% bile salt
MSMC36-9	8.986 ± 0.02	8.973 ± 0.033^{NS}	9.002 ± 0.015^{NS}
MSMC37-3	8.985 ± 0.02	9.007 ± 0.008^{NS}	9.890 ± 0.006^{NS}
MSMC37-4	8.898 ± 0.08	9.023 ± 0.003^{NS}	8.826 ± 0.028^{NS}
MSMC83	8.117 ± 0.01	8.204 ± 0.058^{NS}	8.065 ± 0.042^{NS}

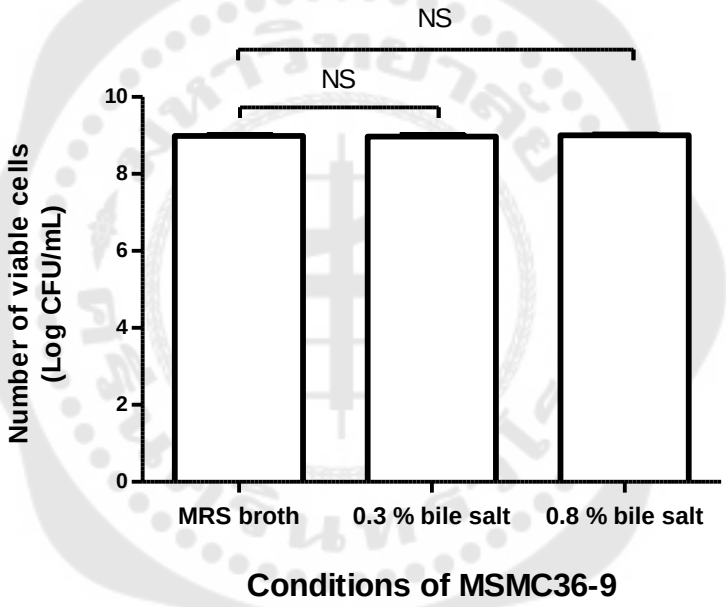

Figure 20 Bile tolerance of MSMC36-9.

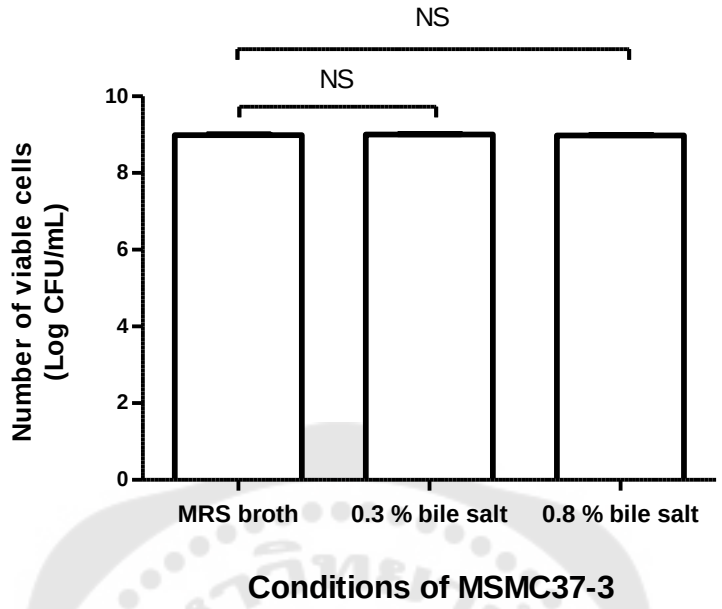

Figure 21 Bile tolerance of MSMC37-3.

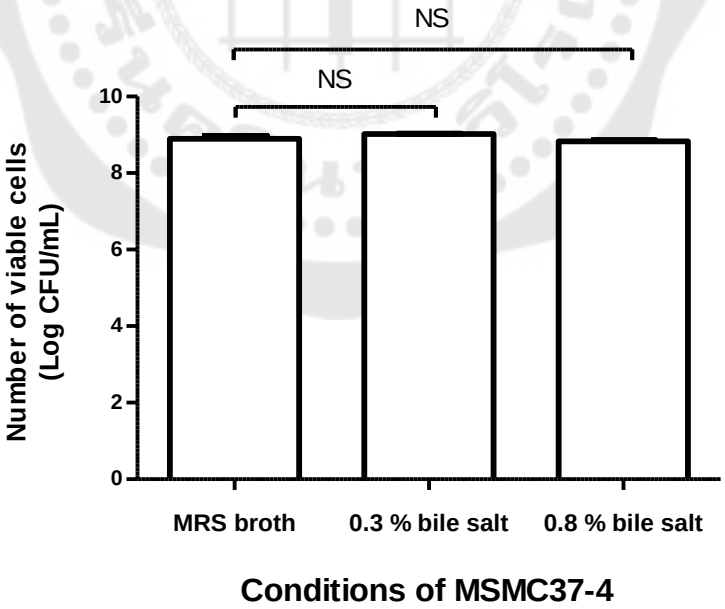

Figure 22 Bile tolerance of MSMC37-4.

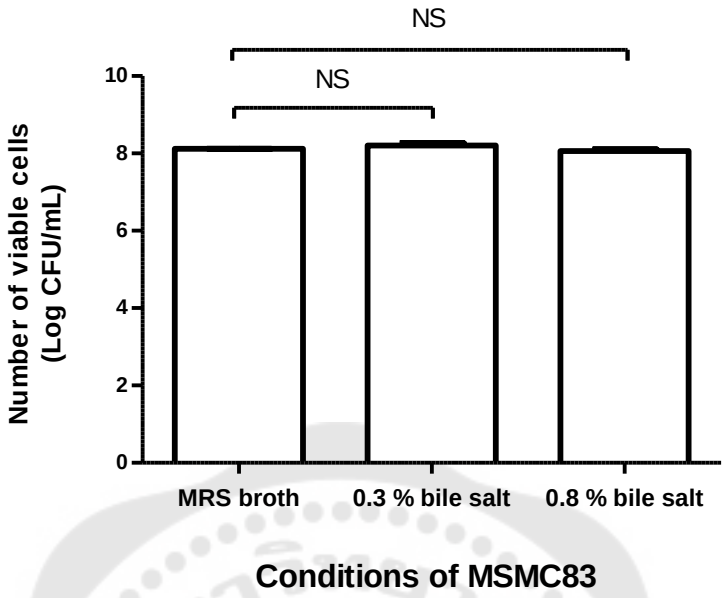

Figure 23 Bile tolerance of MSMC83.

4.3 Adhesion property on Caco-2 cells

Adhesion ability to the intestinal cells is an important selection criterion for potential probiotic. The selected LAB were determined for probiotic property of adherence to Caco-2 cell line. Differences in the apparent adherence of selected LAB ranged from 2.79% to 8.76 % as shown in figure 24. In this study, the ability of adhesion of *L. rhamnoses* GG (positive control) was 3.40% based on their respective adhesion score. The MSMC83 was the most adhesive isolates followed by MSMC36-9, MSMC 37-3, and MSMC37-4, respectively. Comparing with the reference strain *L. rhamnoses* GG, the percentage of adhesion of MSMC36, MSMC37-3, and MSMC83 were significantly higher ($P < 0.05$). There was no significant difference in the adhesion of MSMC37-4 with that of *L. rhamnoses* GG. Therefore, all the selected LAB were strongly able to adhere to the human intestinal cells.

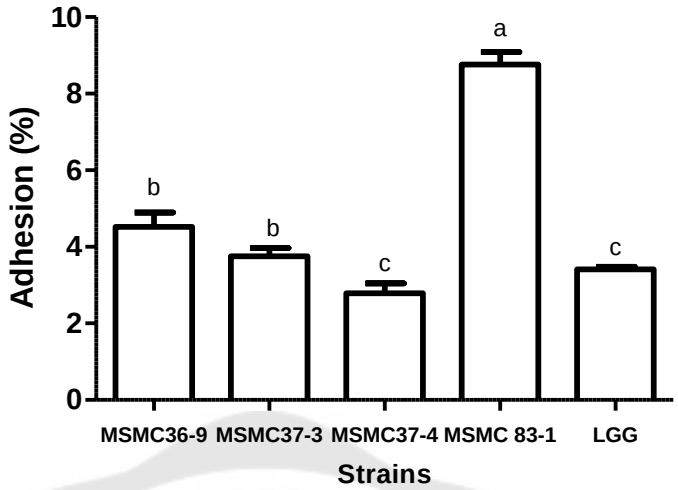

Figure 24 Adhesion ability to Caco-2 cells selected LAB and *L. rhamnosus* GG.

The letters a-c indicates statistically significant differences at $P < 0.05$.

4.4 Hemolytic activity

The results showed that MSMC36-9, MSMC37-3, MSMC37-4 and MSMC83 did not exhibit hemolytic activity (γ-hemolysis). *Streptococcus pyogenes* exhibited complete hemolysis (β-hemolysis) as positive control. Therefore, 4 LAB isolates may be considered as safe with regard to these activities.

4.5 Antibiotic susceptibility testing

The diameter of inhibitory zone of antibiotic susceptibility among the 4 LAB isolates including MSMC36-9, MSMC37-3, MSMC37-4 and MSMC83 (Table 10). All LAB isolates were sensitive to 6 antibiotics including ampicillin, chloramphenicol, erythromycin, tetracycline, bacitracin and streptomycin, whereas it was resistant to vancomycin. Especially, all the strains were sensitive to penicillin G and resistant to gentamycin, nalidixic acid, and vancomycin except MSMC83.

Table 10 Antibiotic susceptibility of selected LAB against the consumption antibiotics.

Antibiotics	Zone of inhibition (mm)			
	MSMC36-9	MSMC37-3	MSMC37-4	MSMC83
Ampicillin	34.50±1.50 (S)	30.75±0.25 (S)	29.75±0.75 (S)	24.60±0.75 (S)
Chloramphenicol	32.25±1.25 (S)	28.50±1.75 (S)	27.25±1.50 (S)	30.25±0.54 (S)
Gentamycin	6.50±1.25 (R)	8.25 ± 0.25 (R)	8.50±0.25 (R)	24.09±0.45 (S)
Nalidixic acid	12.75±1.25 (R)	10.50±1.50 (R)	8.25±0.25 (R)	29.56±0.76 (S)
Penicillin G	34.75±0.50 (S)	30.50±1.50 (S)	32.50±1.75 (S)	9.00±1.50 (R)
Erythromycin	34.75±0.25 (S)	32.75±1.75 (S)	30.25±1.50 (S)	46.00±0.50 (S)
Tetracycline	41.50±1.50 (S)	31.75±1.00 (S)	32.75±0.75 (S)	30.60±0.75 (S)
Vancomycin	8.75±1.25 (R)	8.25±0.75 (R)	6.50±0.25 (R)	9.50±1.50 (R)
Bacitracin	16.25±0.75 (S)	12.25±0.50 (S)	10.25±0.50 (S)	34.60±1.00 (S)
Streptomycin	16.2 ±0.75 (S)	12.25±0.50 (S)	14.50±1.25 (S)	36.0±1.55 (S)

(R): resistant, (S): susceptible.

4.6 Antagonist bacteria

Antibacterial activity test of LAB showed inhibition of zone halo on MH agar plate (Table 11). All isolates showed the inhibition against 10 indicator pathogens using well diffusion agar test. The different scores reflected the different degrees of inhibition expressed in mm. The zones of inhibition ranged from 7.50 mm to 14.25 mm. All LAB isolates showed a moderate zone of inhibition against three pathogens, Methicillin Resistant S. aureus, *M. luteus* and *Proteus mirabilis* while weak zone of inhibition against seven pathogens including *S. aureus, B. subtilis*, *E. coli*, *Shigella dysenteriae*, *V. cholerae, V. parahaemolyticus* and *P. aeruginosa.* Whereas 4 LAB isolates present zero inhibition against *S. pyogenase*, *S. pneumoniae*, *K. pneumonia*, and *Salmonella typhi*.

Table 11 Antimicrobial activity of selected LAB against clinically significant pathogens.

Indicator pathogens	Zone of inhibition (mm)			
	MSMC36-9	MSMC37-3	MSMC37-4	MSMC83
S. aureus	7.50±0.75 (W)	8.00±1.50 (W)	7.50±0.50 (W)	7.50±0.50 (W)
MRSA	11.50±0.25 (M)	10.25±0.75 (M)	10.50±0.25 (M)	10.50±0.50 (M)
S. pyogenase	0.00±0.00 (N)	0.00±0.00 (N)	0.00±0.00 (N)	0.00±0.00 (N)
S. pneumoniae	0.00±0.00 (N)	0.00±0.00 (N)	0.00±0.00 (N)	0.00±0.00 (N)
M. luteus	12.25±0.75 (M)	11.75±0.25 (M)	10.50±0.50 (M)	7.67±0.57 (M)
B. subtilis	6.50±0.75 (W)	7.50±0.50 (W)	6.50±0.75 (W)	7.33±0.57 (W)
E. coli	7.50±0.50 (W)	6.50±0.75 (W)	6.00±1.50 (W)	8.00±1.00 (W)
K. pneumonia	0.00±0.00 (N)	0.00±0.00 (N)	0.00±0.00 (N)	0.00±0.00 (N)
P. mirabilis	12.00±0.75 (M)	12.00±1.50 (M)	12.50±0.75 (M)	14.25±0.75 (M)
Salmonella typhi	0.00±0.00 (N)	0.00±0.00 (N)	0.00±0.00 (N)	0.00±0.00 (N)
Shigella dysenteriae	7.50±0.75 (W)	6.50±0.75 (W)	8.00±0.75 (W)	7.67±0.57 (W)
V. cholerae	8.25±0.50 (W)	8.50±1.50 (W)	8.750±0.50(W)	7.33±0,76 (W)
V. parahaemolyticus	9.25±0.75 (W)	7.50±1.25 (W)	8.50±1.25 (W)	7.50±0.76 (W)
P. aeruginosa	8.50±1.50 (W)	9.50±0.25 (W)	7.25±1.50 (W)	8.67±0.57 (W)

The different scores reflect the different degree of growth inhibition expressed in mm (n=3, mean ± SD), diameter of well was designed at 4.0 mm. (N), no inhibition; (W), weak zone of inhibition between 4 and 10 mm; (M), moderate zone of inhibition between 10.1 and 20 mm; (S), strong zone of inhibition ≥ 20.1 mm.

5. Genotypic identification of selected probiotic lactic acid bacteria

Four selected LAB with antioxidant activity were identified by 16S rRNA gene sequencing. Comparative analysis of 16S rRNA gene sequences confirmed the results from the cluster analysis and revealed that selected LAB were 100% and 99% relatively similar to the genus *Lactobacillus* and *Bifidobacterium*, respectively. The results showed that the MSMC36-9, MSMC37-3 and MSMC37-4 belonged to *Lactobacillus paracasei* JCM 1171^{T} and the MSMC83 exhibited 99.20% similarity scores to *Bifidobacterium animalis* DSM 10140^{T} (Table 12).

Table 12 Genotypic identification of selected LAB on 16S rRNA gene sequencing.

Isolate no.	Closest strain	Similarity (%)	Length (bp)
MSMC36	*Lactobacillus paracasei* JCM 1171^{T}	100.00	1,388
MSMC37-3	*Lactobacillus paracasei* JCM 1171^{T}	100.00	1,369
MSMC37-4	*Lactobacillus paracasei* JCM 1171^{T}	100.00	1,466
MSMC83	*Bifidobacterium animalis* DSM 10140^{T}	99.20	1,388

A phylogenetic tree was constructed based on their 16S rRNA gene sequences utilizing the neighboring method. The MSMC36-9, MSMC37-3 and MSMC37-4 exhibited closest relatives among the genus *Lactobacillus* while MSMC83 showed cluster of *Bifidobacterium* (Figure 25).

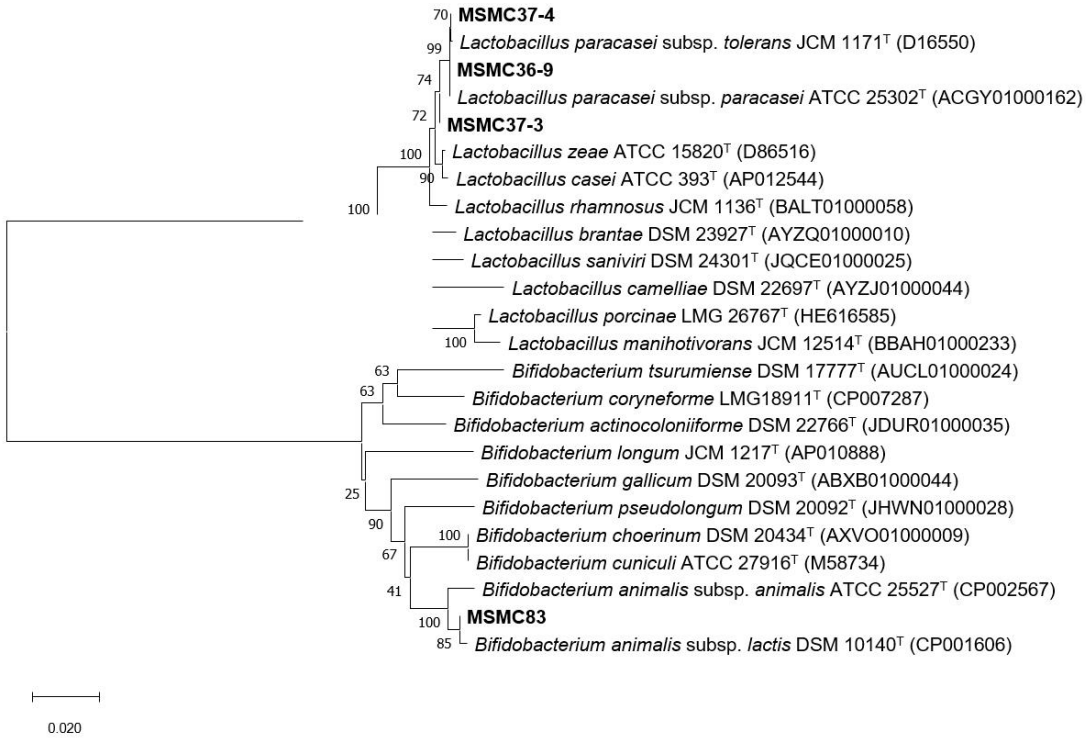

Figure 25 Phylogenetic tree based on 16S rRNA gene sequences of isolates.

6. Antioxidant effects of the *B. animalis* MSMC83 on D-galactose induced rats

B. animalis MSMC83 was found to have higher antioxidant potential hence was selected for antioxidant effect on D-galactose induced rats.

6.1 Changes of body weight, and organ Index

The body weight and the organ including liver, spleen, stomach, and total fat of rat was weighed and recorded. There were no deaths during the experiment. The body weight of rat given *B. animalis* MSMC83 or ascorbic acid were significantly decreased, compared with D-gal induced group ($P < 0.05$) as show table 7.

The liver index of D-gal induced group increased significantly as compared with normal group ($P < 0.05$), while *B. animalis* MSMC83 or ascorbic acid treated rat decreased significantly ($P < 0.05$) compared to D-gal induced group, and there was no significant difference compared to normal group (Table 13). However, there was no significant different in spleen and stomach. The total fat index of probiotic and ascorbic acid groups decreased significantly ($P < 0.05$) as compared with D-gal induced group. Thus, *B. animalis* MSMC83 decreased weight of body, liver, and total fat on D-galactose induced oxidative stress.

Table 13 Effect of *B. animalis* MSMC83 on the organs weight.

Index	Body weight	Liver	Spleen	Stomach	Total fat
Normal	600.60±8.79	14.90±0.35	0.82±0.05	2.75±0.17	38.11±0.64
D-Gal model	635.67±10.97	17.05±1.37$^{\#}$	0.93±0.03	2.91±0.07	42.81±0.57$^{\#}$
Probiotic	573.65±16.44*	14.03±0.34*	0.77±0.05	3.13±0.27	34.03±1.23*
Ascorbic acid	576.69±31.6*	15.08±0.49*	0.83±0.13	2.72±0.60	34.56±1.73*

Each value is expressed as mean ± SD. * means significantly different compared to D-gal induced group ($P < 0.5$) and # means significantly different compared to normal group ($P < 0.5$).

6.2 Changes of biochemical in serum

The biochemical including fasting blood glucose, lipid profiles, and liver function test, in serum of different groups after feeding were shown in table 14. A significant increase of glucose, total cholesterol triglyceride and aspartate aminotransferase (AST) levels was observed in D-gal induced group compared to normal group ($P < 0.05$). It was found that glucose, total cholesterol, triglyceride levels, and aspartate aminotransferase (AST) level in serum were decreased significantly after feeding *B. animalis* MSMC83 and ascorbic acid compared to model group ($P < 0.05$). Thus, *B. animalis* MSMC83 provides a decreased level of glucose, total cholesterol triglyceride and AST on D-galactose induced oxidative stress.

Table 14 Effect of *B. animalis* MSMC83 on biochemical in serum.

Biochemical	Normal	D-Gal induced	Probiotic	Ascorbic acid
Glucose	203.67±1.53	221.33±5.13#	105.00±3.46*	172.67±6.43*
Total cholesterol	49.33±3.22	60.67±0.58#	50.33±1.53*	50.67±2.08*
Triglyceride	84.33±3.79	229.67±7.57#	128.00±5.57*	102.00±5.20*
HDL	31.33±5.03	32.33±1.53	35.67±0.58	30.33±1.53
LDL	5.00±1.00	5.33±0.58	5.67±0.58	6.67±0.58
AST	74.00±4.58	90.67±5.77#	68.07±3.90*	70.37±9.02*
ALT	26.67±0.58	28.33±4.04	25.67±3.06	27.77±1.55
ALP	50.33±5.51	43.33±1.53	48.67±1.53	44.67±2.56

Each value is expressed as mean ± SD. * means significantly different compared to D-gal induced group ($P < 0.5$) and # means significantly different compared to normal group ($P < 0.5$).

6.3 Effect of *B. animalis* MSMC83 on antioxidant and lipid peroxidation level in serum

Antioxidant enzymes in serum was detected by superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), catalase. In this study, D-gal model group displayed significantly decreased activities of antioxidant enzymes in serum. As shown in table 15, the antioxidant ability of feeding with *B. animalis* MSMC83 was 205.87±17.25 U/mL, 883.20±14.62 U/mL, 74.93±2.56 U/mL of the level of T-SOD, GSH-Px, and catalase, respectively. The *B. animalis* MSMC83 treated rats exhibited improvement in T-SOD, GSH-Px, and catalase in serum. When compared with the D-gal model group, the *B. animalis* MSMC83 significantly increased the activities of T-SOD (Figure 26), GSH-Px (Figure 27), and catalase (Figure 28) in serum ($P < 0.05$), which was similar to ascorbic acid treated oxidative stress group. Therefore, *B. animalis* MSMC83 demonstrated the same potential for enhancing antioxidant capacity in D-Gal induced oxidative stress by raising antioxidant enzymes.

Malondialdehyde (MDA), a final product of lipid peroxidation by free radicals, is a powerful biological indicator for oxidative stress.[169] Induced by D-galactose in rat was successfully presented because serum lipid peroxidation levels of the D-gal model group were significantly higher than the normal group (Figure 29). The level of MDA in serum of probiotic group and ascorbic acid were 4.56±1.37 nmol/ml and 5.23±1.06 nmol/ml, respectively. The ability of *B. animalis* MSMC83 to decrease level of MDA in serum was significantly compared to model group ($P < 0.05$). Furthermore, *B. animalis* MSMC83 reduced lipid peroxidation levels as well as the positive group and returned to normal levels.

Table 15 Effect of *B. animalis* MSMC83 on antioxidant and lipid peroxidation level in serum.

Groups	Antioxidant activity			
	T-SOD (U/mL)	GSH-Px (U/mL)	Catalase(U/mL)	MDA (nmol/ml)
Normal	178.1333±9.22***	820.00±15.26***	58.99±2.23***	4.99±1.61***
D-gal model	127.7333±15.79	705.07±17.65	47.73±3.45	12.40±1.64
Probiotic	205.87±17.25***	883.20±14.62***	74.93±2.56***	4.56±1.37***
Ascorbic acid	176.53±11.34***	842.93±20.47***	68.00±2.11***	5.23±1.06***

Normal group, D-gal model group, Probiotic group: feeded with *B. animalis* MSMC83, Ascorbic acid: feeded with ascorbic acid. Data are presented as means ± SD. *** $P < 0.001$ compared with D-gal model group (n = 5).

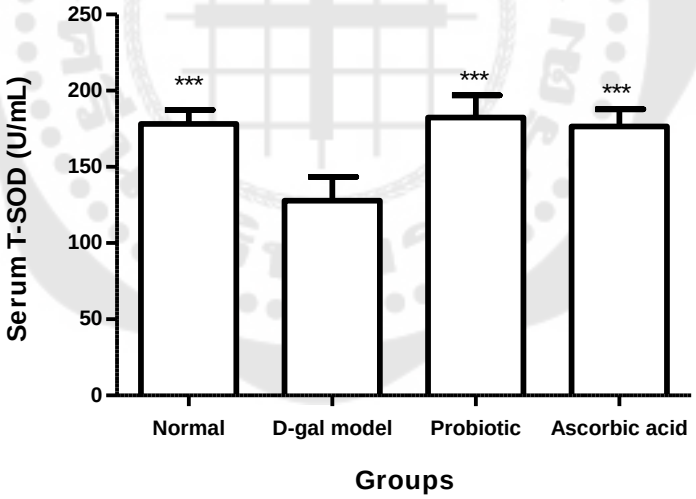

Figure 26 Effects of *B. animalis* MSMC83 on serum total superoxide dismutase.

The data are presented as means ± SD (n = 5). *** $P < 0.001$: significantly different compared with D-gal model group.

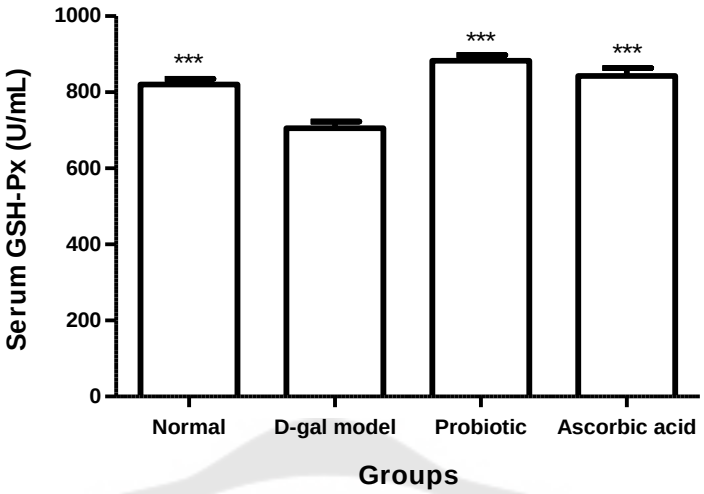

Figure 27 Effects of *B. animalis* MSMC83 on serum glutathione peroxidase.

The data are presented as means ± SD (n = 5). *** $P < 0.001$: significantly different compared with D-gal model group.

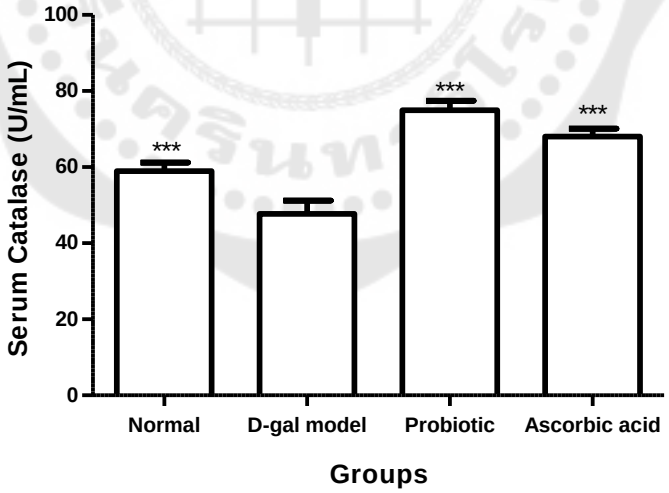

Figure 28 Effects of *B. animalis* MSMC83 on serum catalase.

The data are presented as means ± SD (n = 5). *** $P < 0.001$: significantly different compared with D-gal model group.

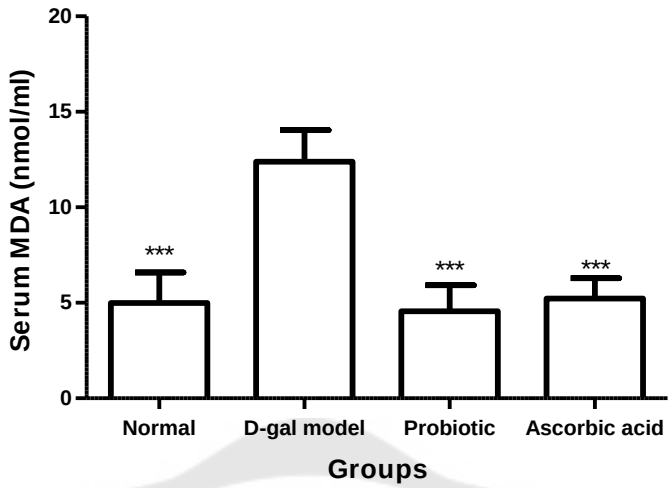

Figure 29 Effects of *B. animalis* MSMC83 on serum malondialdehyde level.

The data are presented as means ± SD (n = 5). *** $P < 0.001$: significantly different compared with D-gal model group.

6.4 Effect of *B. animalis* MSMC83 on TNF-alpha levels in liver

The level of tumor necrosis factor (TNF)-**α**, pro-inflammatory cytokines expressed in the liver of D-gal induced rats obviously increased to 109.60 ± 5.11 pg/mL when compared with normal group (Table 16). Thus, D-galactose administration produced significant liver chronic inflammation in rats. Interestingly, our study showed that eight weeks of treatment with *B. animalis* MSMC83 was significantly reduced the expression of pro-inflammatory cytokines, compared with D-gal model group ($P > 0.05$) as show in Figure 30. Therefore, *B. animalis* MSMC83 administration significantly reduced inflammation of the liver caused by D-gal induced oxidative stress.

Table 16 Effect of *B. animalis* MSMC83 on tumour necrosis factor – α in liver.

Groups	Tumor necrosis factor - α (pg/mL)
Normal	57.87±4.86***
D-gal model	109.60±5.11
Probiotic	96.27±4.04***
Ascorbic acid	70.67±4.22***

Normal group, D-gal model group, Probiotic group: feeded with *B. animalis* MSMC83, Ascorbic acid: feeded with ascorbic acid. Data are presented as means ± SD. *** $P < 0.001$ compared with D-gal model group (n = 5).

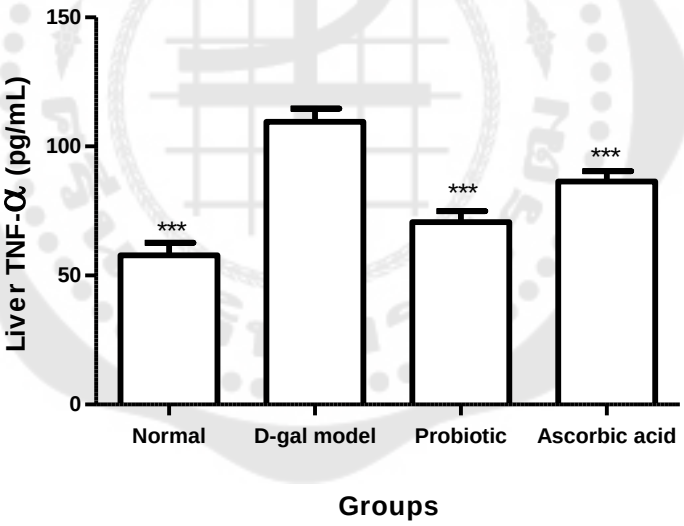

Figure 30 Effect of *B. animalis* MSMC83 on tumour necrosis factor – α in liver.

The data are presented as means ± SD (n = 5). *** $P < 0.001$: significantly different compared with D-gal model group.

6.5 Changes of intestinal microbiota

Fecal samples in all groups were collected and were counted by spread plate on eosin methylene blue agar (EMB) agar, *Bifidobacterium* selective media (BSM) agar, and MRS with $CaCO_3$.

Fecal *E. coli* colonies was detected on EMB agar and was confirmed by Gram stain as show in Figure 31. *Escherichia coli* in fecal count between normal and D-gal induced groups were significantly differenced ($P < 0.05$). As compared with D-gal induced group, *E. coli* count significantly decreased in probiotic group that ($P < 0.05$). In this case, *E. coli* counts did return to control in the probiotic group, while enlarged in the ascorbic acid group. Therefore, probiotic exposure resulted the reduction of pathogens in the intestines induced oxidative stress.

Fecal Bifidobacteria were counted on BSM agar supplement with BSM selective. Bifidobacteria counts significantly decreased in the D-induced model group compared with the normal group ($P < 0.05$), while it significantly increased in the administeration with *B. animalis* MSMC83 (Figure 32). Therefore, *B. animalis* MSMC83 exposure can increase the amount of bifidobacteria and can stability in the intestinal induced oxidative stress.

Fecal LAB colonies were screened when clear zones were shown in MRS with $CaCo_3$. The LAB count was 8.45 ± 0.18 CFU/g in D-induced model group, significantly raised compared with normal group ($P < 0.05$) as show in Figure 33. Interestedly, the amount of LAB in probiotic and ascorbic acid groups were similar to the normal group ($P > 0.05$). Therefore, exposure to *B. animalis* MSMC83 may increase the amount of LAB in the intestinal induced oxidative stress group.

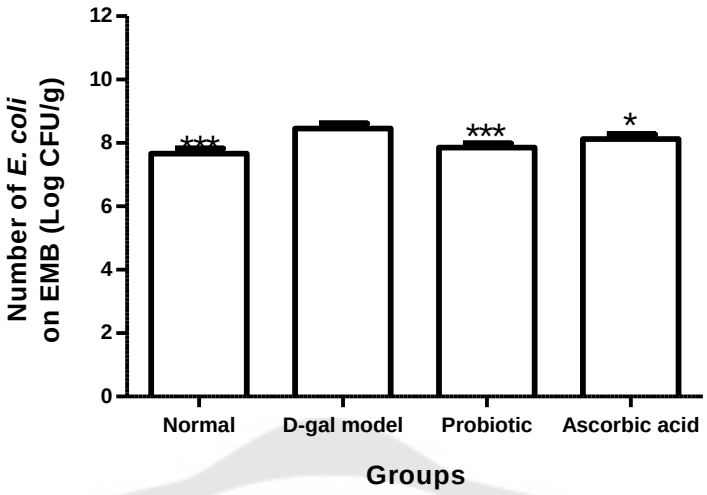

Figure 31 Average faecal *E. coli* count on EMB agar in rat administered with *B. animalis* MSMC83.

Statistical tests were performed using paired t-test. The data are presented as means ± SD (n = 5). * $P < 0.05$, *** $P < 0.01$: significantly different compared with D-gal model group.

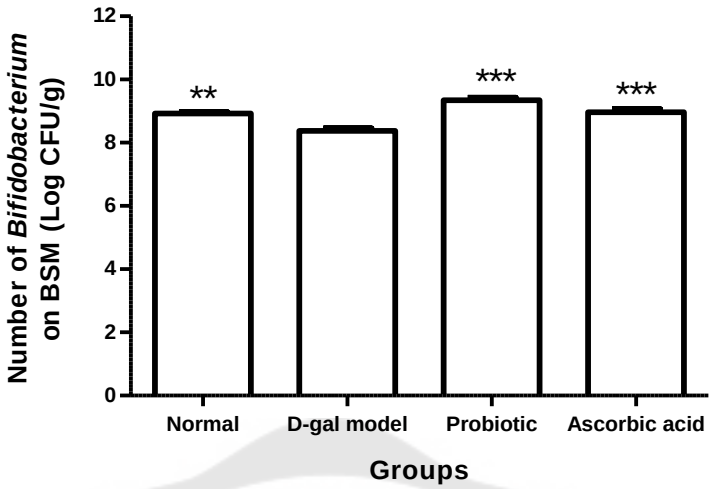

Figure 32 Average faecal *Bifidobacterium* count on BSM agar in rat administered with *B. animalis* MSMC83.

Statistical tests were performed using paired t-test. The data are presented as means ± SD (n = 5). ** $P < 0.01$, *** $P < 0.001$: significantly different compared with D-gal model group.

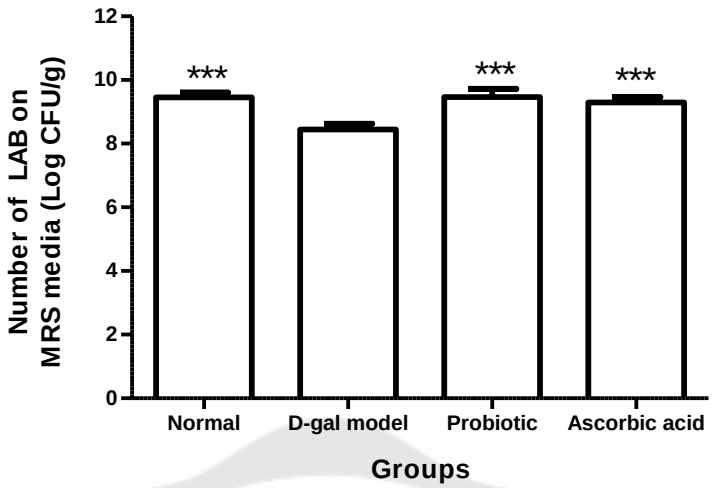

Figure 33 Average faecal lactic acid bacteria count on MRS agar in rat administered with *B. animalis* MSMC83.

Statistical tests were performed using paired t-test. The data are presented as means ± SD (n = 5). *** $P < 0.001$: significantly different compared with D-gal model group.

7. Measurement for chemical and physical properties of milk fermented with selected probiotic lactic acid bacteria.

Bifidobacterium animalis MSMC83 had antioxidant activity *in vitro* and *in vivo.* In addition, a survival study of *B. animalis* MSMC83 in milk incubated at 43 °C showed a survival rate greater than 90 % after 6 h incubation. Thus, *B. animalis* MSMC83 was selected for development of yogurt products. The microbiology and physicochemical properties of milk fermented with *B. animalis* MSMC83 strains combined with starter cultures were evaluated weekly during 4 weeks of storage at 4±1 ˚C.

7.1 Preparation of starter cultures and fermentation of milk

The pH profiles of the yoghurts fermented by regular yoghurt starter cultures as a control sample and yoghurt fermented by starter cultures combined with *B. animalis* MSMC83 are shown in Figure 34. Fermentation was stopped at pH 4.6 by transferring the samples to a refrigerator. The fermentation time was 6 h for the control sample, and 4 h 30 min for probiotic yoghurt.

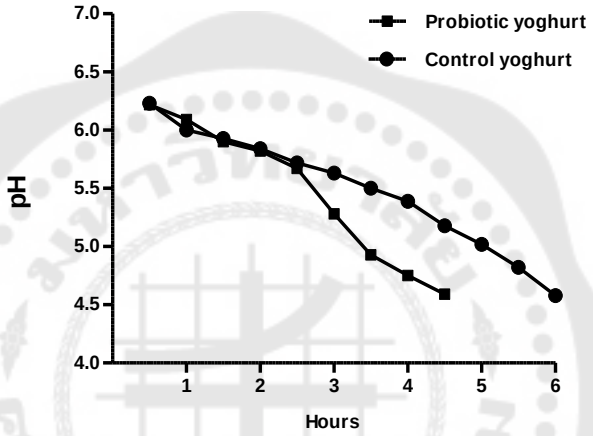

Figure 34 Fermentation time of control yoghurt (starter cultures) and probiotic yoghurt (starter cultures + *B. animalis* MSMC83).

7.2 Measurement of viability

The inoculum size of *L. bulgaricus, S. thermophilus*, and *B. animalis* MSMC83 were about 1×10^9 CFU/mL. The viable counts of starter cultures and probiotic during storage are presented on Table 17. On Day1, the mean numbers of viable cells of bacteria in probiotic yoghurt were 9.24 ± 0.02 log CFU/g, while the control yoghurt had 9.16 ± 0.04 log CFU/g (Table 2). During 4 weeks of storage, the bacterial counts were constant in the control sample. The bacterial counts in probiotic yoghurt were significantly decreased in the 4^{th} week ($P < 0.05$).

Table 17 Viability of control yoghurt and probiotic yoghurt during 4 weeks of storage at 4±1°C.

Yoghurts	Viability of microorganism (Log CFU/g)				
	Initial	Week 1	Week 2	Week 3	Week 4
Control	9.16 ± 0.04^{Ac}	9.21 ± 0.05^{Bc}	9.51 ± 0.03^{Bb}	9.81 ± 0.06^{Aa}	9.84 ± 0.10^{Aa}
Probiotic	9.24 ± 0.02^{Ac}	9.32 ± 0.03^{Ac}	9.65 ± 0.07^{Ab}	9.85 ± 0.09^{Aa}	9.60 ± 0.09^{Ba}

Each value is the mean ± SD of three independent readings. The different superscript letters a-c in the same row and letters A-B in the same column represent statistical significance ($P < 0.05$).

7.3 Measurement of pH

The pH levels of yoghurts during storage are shown in Table 18. The average pH values for probiotic yoghurt ranged from 4.67 to 3.72 during the storage. A consistent decrease in pH was found during 4 weeks of storage ($P < 0.05$).

Table 18 pH value of control yoghurt and probiotic yoghurt during 4 weeks of storage at 4±1°C.

Yoghurts	pH value				
	Initial	Week 1	Week 2	Week 3	Week 4
Control	4.59 ± 0.03^{Ac}	4.43 ± 0.03^{Bb}	4.23 ± 0.04^{Ba}	4.24 ± 0.02^{Ba}	4.27 ± 0.05^{Ba}
Probiotic	4.67 ± 0.04^{Be}	4.32 ± 0.02^{Ad}	3.84 ± 0.04^{Ab}	3.72 ± 0.05^{Aa}	4.09 ± 0.05^{Ac}

Each value is the mean ± SD of three independent readings. The different superscript letters a-e in the same row and letters A-B in the same column represent statistical significance ($P < 0.05$).

7.4 Measurement of texture

Texture firmness of both yoghurts was measured during storage and the results were presented in Table 19. During storage, addition of *B. animalis* MSMC83 with starter culture significantly increased firmness ($P < 0.05$) in the probiotic yoghurt.

Table 19 Firmness properties of control yoghurt and probiotic yoghurt during 4 weeks of storage at 4±1˚C.

Yoghurts	Firmness (g)				
	Initial	Week 1	Week 2	Week 3	Week 4
Control	85.75 ± 0.77^{Bc}	87.80 ± 0.84^{Bb}	95.10 ± 0.42^{Ba}	72.35 ± 0.77^{Bd}	65.85 ± 0.56^{Be}
Probiotic	106.56 ± 0.79^{Ae}	114.90 ± 0.42^{Ad}	136.25 ± 0.91^{Ab}	147.55 ± 0.49^{Aa}	120.30 ± 0.14^{Ac}

Each value is the mean ± SD of three independent readings. The different superscript letters a-e in the same row and letters A-B in the same column represent statistical significance ($P < 0.05$).

7.5 Measurement of syneresis

The results of syneresis measurement of both yoghurts during storage presented in Table 20. The syneresis values of both yoghurts significantly increased with storage time ($P < 0.05$). The addition of *B. animalis* MSMC83 in probiotic yoghurt significantly decreased the syneresis compared to the control yoghurt.

Table 20 Syneresis properties of control yoghurt and probiotic yoghurt during 4 weeks of storage at 4±1°C.

Yoghurts	Syneresis (%)				
	Initial	Week 1	Week 2	Week 3	Week 4
Control	8.99 ± 0.87^{Bd}	15.88 ± 0.35^{Bc}	19.79 ± 0.25^{Bb}	21.58 ± 0.52^{Ba}	22.46 ± 0.38^{Ba}
Probiotic	1.33 ± 0.41^{Ad}	2.56 ± 0.33^{Ac}	3.26 ± 0.18^{Ac}	4.69 ± 0.15^{Ab}	4.99 ± 0.10^{Aa}

Each value is the mean ± SD of three independent readings. The different superscript letters a-d in the same row and letters A-B in the same column represent statistical significance ($P < 0.05$).

7.6 Rheological properties

The storage modulus (G') and the loss modulus (G") at frequency of 1 Hz are displayed in Table 21-22. G' values were higher than G" values in both yoghurts across the entire measuring range. In probiotic yoghurt, the maximum G' value was observed at 4th week with the value of 482.767 ± 3.65 Pa and the weakest gel with the G' value of 270.17 ± 5.71 Pa observed during initial week of storage. As compared to the control yoghurt, G' and G" of probiotic yoghurt were significantly higher ($P < 0.05$).

Table 21 Storage modulus of control yoghurt and probiotic yoghurt during 4 weeks of storage at 4±1˚C.

Yoghurts	Storage modulus (G') (Pa)				
	Initial	Week 1	Week 2	Week 3	Week 4
Control	161.53±2.55^{Be}	222.17±6.57^{Bd}	246.60±6.66^{Bc}	287.20±1.57^{Bb}	327.08±2.66^{Ba}
Probiotic	270.17±5.71^{Ae}	350.17±10.32^{Ac}	383.27±8.06^{Ac}	420.67±5.40^{Ab}	482.77±3.65^{Aa}

Each value is the mean ± SD of three independent readings. The different superscript letters a-e in the same row and letters A-B in the same column represent statistical significance ($P < 0.05$).

Table 22 Loss modulus of control yoghurt and probiotic yoghurt during 4 weeks of storage at 4±1˚C.

Yoghurts	Loss modulus (G") (Pa)				
	Initial	Week 1	Week 2	Week 3	Week 4
Control	44.835±4.41^{Be}	58.37±0.69^{Bd}	68.32±0.72^{Bc}	81.26±1.03^{Bb}	92.69±0.46^{Ba}
Probiotic	66.46±3.37^{Ac}	81.37±0.86^{Ab}	95.24±4.09^{Aa}	105.96±8.16^{Aa}	116.84±4.60^{Aa}

Each value is the mean ± SD of three independent readings. The different superscript letters a-d in the same row and letters A-B in the same column represent statistical significance ($P < 0.05$).

7.7 Apparent viscosity

As shown in Table 23, the change in viscosity in both yoghurts during 4 weeks of storage was not significant, although the viscosity values slightly increased. However, the probiotic yoghurt was more viscous than the control yoghurt during refrigeration storage.

Table 23 Apparent viscosity of control yoghurt and probiotic yoghurt during 4 weeks of storage at 4±1˚C.

Yoghurts	Apparent viscosity (mPa·s)				
	Initial	Week 1	Week 2	Week 3	Week 4
Control	84.12±5.46^{Ba}	90.74 ±7.26^{Ba}	101.36±5.27^{Ba}	104.47±0.46^{Ba}	105.47±1.47^{Ba}
Probiotic	138.53±13.20^{Aa}	151.20±19.70^{Aa}	185.20±06.20^{Aa}	190.63±17.75^{Aa}	180.47±28.17^{Aa}

Each value is the mean ± SD of three independent readings. The different superscript letters a-b in the same row and letters A-B in the same column represent statistical significance ($P < 0.05$).

CHAPTER V
DISCUSSION

The free radicals are highly unstable biomolecules and can be divided into three groups consist of reactive oxygen species (ROS), reactive nitrogen species (RNS), and reactive chlorine species (RCS).[16] Reactive oxygen species (ROS), including hydroxyl radical ($HO^{\cdot}$), hydrogen peroxide (H_2O_2), and superoxide radicals (O_2^{-}), are the most important forms of free radicals and generated continuously in the mitochondria of growing cells.[18] Among them, hydroxyl radical is the most dangerous free radical because it directly damages DNA, proteins, and lipids.[170] Malondialdehyde (MDA) is the end product of ROS-induced lipid peroxidation and is the most commonly used biomarkers to reflect lipid peroxidation levels of oxidative stress.[169, 171, 172] Additionally, ROS cause oxidative stress and lead to many disease.[173-175] Antioxidants, synthesized from cells and obtained from foods, reduce free radical and prevent oxidative stress.[21] Therefore, the increase in antioxidants is considered vital in the reduction of oxidative damage and the risk factor of diseases.[176, 177]

The most commonly utilized lactic acid bacteria (LAB) as probiotic microorganisms include *Bifidobacterium,* and *Lactobacillus.*[178] In the human body, probiotic can adhere and colonize in the human gastrointestinal tract and tolerate low pH and bile salt conditions as normal microbiota.[179] Probiotics are diverse and have different beneficial properties on human health depending on the specific characteristics of each strain.[180] Some probiotic strains have shown to possess antioxidative activity, and reduce free radical accumulation by modulating the redox status of the host via their antioxidant system.[11, 123]

In this study, 2 free radical scavenging activity assays were used as suggested by Mishra et al. (2015)[181] because they are simple, quick and repeatable approach.[182] DPPH radical scavenging activity, and hydroxyl free radical scavenging activity were used for the measure of the antioxidant activity of 50 lactic acid bacteria isolates. The free radical scavenging activity of DPPH is due to its ability to donate electrons or

hydrogen.[183] Whereas, hydroxyl radicals are the most reactive radicals and can lead to lipid peroxidation, as well as interaction with other biomolecules of the cells.[184] Four LAB isolates including MSMC36-9, MSMC37-3, MSMC37-4, and MSMC83, were found to have antioxidant property. The results of the two radical scavenging methods showed that the viable cells, cell free supernatant and intracellular cell free extract of four LAB isolates exhibited antioxidant activity. The scavenging activity of intracellular cell free extract (ICFE) was significantly higher than that of viable cells and cell free supernatants (CFS) ($P < 0.05$). The results revealed that four LAB isolates possibly contained some potential bioactive compounds, which were electron donors and effectively react with free radicals to convert them to more stable products and terminate the radical chain reaction.[16] The previous studies showed that some LAB strains have antioxidant activity.[185] The observation by Mu G et al. (2018) showed that 13 of LAB strains exhibited DPPH scavenging activity.[186] *Lactobacillus fermentum*, *L. paracasei* and *L. rhamnosus* strains were were high in DPPH radical scavenging activity high DPPH radical scavenging than *L. salivarius, L. oris* and *L. gasseri* strains.[187] Viable cells and ICFE of *L. casei* SY13 and *L. delbrueckii* LJJ had different capacities on DPPH radical and hydroxyl radical scavenging activities.[127] Similarly, Kim et al. (2003) reported that 25 *Bifidobacterium* strains exhibited DPPH radical scavenging activity ranged from 15 to 41 %, which was close to our findings.[188] Shen S et al. (2011) indicated that intact cells, culture supernatant, and ICFE of *B. animalis* 01 showed strong scavenging effect of DPPH radical scavenging activity and hydroxyl radical scavenging activity.[125] Therefore, the antioxidant activity of probiotics depends highly on strains.[187] Moreover, the ICFE exhibited the highest antioxidant activity than intact cells and CFS. It may indicate that the antioxidation factor highly present in the cells and the short reaction time may affect the antioxidant capacity of intact cells and CFS.[11] In hydroxyl radical scavenging activity assay, hydroxyl radical generated from the reaction between ferrous iron and hydrogen peroxide (the Fenton reaction).[189] Therefore, 4 LAB isolates may inhibit hydroxyl radical formation via iron chelation in a Fenton reaction.[126]

In this study, the antioxidant activity of viable cells, CFS and ICFE of 4 LAB isolates inhibited intracellular ROS in Caco-2 cells. The ICFE showed significantly higher antioxidant activity than the viable cells and CFS ($P < 0.05$). The ICFE of MSMC83 showed maximal antioxidant activity value of 80.23% compared to other LAB isolates. The different mechanisms involved in the radical antioxidant reactions are dependent on species and strains of probiotic and host cells. The composition of antioxidants can be transported to cells differently.[152] Antioxidative activities of probiotic strains showed an effective protection against oxidative damage in Caco-2 cells.[186] Xing et al. (2015) reported that the antioxidant activity of CFS is greater than viable cells in Caco-2 cells.[41] Lin et al. (2000) found similar results with the ICFE of *B. longum* and *L. bulgaricus* exhibiting a higher inhibitory effect of the H_2O_2-induced oxidative damage in intestinal cells, compared with the intact cells.[96] The antioxidant factor of ICFE effect more than the released by intact cells.[40] Our results clearly indicated that inhibition of the production of ROS by probiotics in intestinal cells induced by H_2O_2 can be achieved and used to formulate functional foods to prevent intestinal cells from the damage caused by oxidative stress.

As we all know, probiotics can be found in the human gastrointestinal tract.[190] Probiotics need to be resistant to low pH gastric juice and to tolerate high concentrations of bile salts in the gut to increase survival.[191] The gastric juice is secreted into the stomach for approximately 90 minutes, and then through a digestion process which takes longer times.[192] The pH of the gastric juice is pH 2.0 and increases to 3.0 when there is food.[193] After probiotics have pass through the stomach, they enter the duodenum where bile is secreted. The duodenum, upper small intestine, has the concentration of bile salts in the range of 0.3-0.8%.[194] The ability to tolerate bile for survival during human gastrointestinal tract is an important factor. Acid and bile tolerance of probiotics depend on strains and related to their biological characteristics. In this study, 4 LAB isolates survived in pH 3.0 and 4.0 after 3 h exposure. Interestingly, MSMC83 showed the survival rate of 72.75% at pH 2.0 and higher at pH 3.0 and 4.0. Moreover, survival rate of 4 LAB isolates exposed in 0.3%, and 0.8% (w/v) bile salt after

3 h exceeded 99%. *L. fermentum* N2, *L. plantarum* N15, *L. rhamnosus* N22, and *L. paracasei* N20 isolated from feces of infants in southwest Iran reported by Jomehzadeh N et al. (2020), were survived acid conditions at pH 2.5 after 2 h of incubation.[195] Qureshi N et al. (2020) found the survival ability of *L. paracasei* ZFM54 showed tolerance and stability to simulated gastric acid at pH 3.0.[196] Among 545 strains, ten of *Bifidobacterium* strains were the strongest acid tolerance and exhibited nearly 100% viability under pH 2 for 2 h.[197] These results are similar to the results obtained by Mangia et al. (2018), who found that *L. paracasei* FS103 were able to survive in gastric and intestinal juice.[198] *L. paracasei* LBC 81 had stability of survival in 0.3% bile salts the course of 4 h of incubation. [173] Moreover, *L. fermentum*, *L. plantarum* and *L. paracasei* DUR 18 exhibited more than 90% of the survival rate at 0.3% oxgall.[195] According to Zhang et al. (2016), survival rate of *Bifidobacterium* spp. was 93-98% at pH 3 and 61-98% at 0.3% bile salt. Our results indicated that 4 LAB isolates were able to survive under gastric juice and intestinal juice.[199] In this study, 4 LAB isolates also present well adhesion to Caco-2 cells. The ability to adhere to Caco-2 cells of 4 LAB isolates were observed range from 2.79% to 8.76 %. Comparing with the reference strain *L. rhamnoses* GG, the 4 LAB isolates had higher percentages of adhesion ($P < 0.05$). A previous study reported that the adhesion ability of *L. paracasei* 62LP39 isolated from Sardinian dairy products was the most adhesive strain.[200] Additionally, the capability of adhesion was dependent on the individual LAB strain.[201] *Bifidobacterium* isolated from subjects of different ages also resulted in different degrees of adhesion.[202] Due to aging, some of the properties necessary for their adhesion are lost. *Bifidobacterium* isolated from elderly subjects had less ability than isolated from infant.[203] In addition, the ability of LAB to adhere to Caco-2 cells is correlated to the cell surface charge. Bacterial cell wall with cell surface hydrophobicity results in increased adhesion capacity. [204] In addition, the LAB binding mechanism is associated with a receptor-specific bind to intestinal cells.[205] Protein binding on cell surface of LAB such as cell and mucus-binding protein A (CmbA), mucus-binding protein (Mub) and mucus adhesion-promoting protein (MapA), are differentiated, which

promote cell adhesion to human intestinal cell.[205, 206] Additionally, the species may exhibit a degree of specificity to receptor intestinal epithelial cell membrane of the host for adhesion.[207, 208] There are several advantages of adhesion, such as activating colonization, increasing substance production and compete for the island area of pathogens which plays several protective roles.[209] Therefore, 4 LAB isolates in this study are a potential probiotic strains to grow and function in gastrointestinal tract.

The hemolytic assessment of bacteria is critical as a criterion for the selection of bacteria in food products.[210] In this study, 4 LAB isolates showed α- hemolytic and β-hemolytic activity when grown in brain heart infusion agar supplemented with 5 % (v/v) sheep blood. Several studies have shown that probiotics have not been shown hemolytic activity, especially *Lactobacillus* and *Bifidobacterium*.[211]

Previous studies reported that *Lactobacillus* and *Bifidobacterium* generally sensitive to ampicillin, chloramphenicol, penicillin, erythromycin, and tetracycline.[212] penicillin G, vancomycin, tetracycline and erythromycin were chosen because Gram-positive anaerobes are frequently resistant to these antibiotics.[213] Four LAB isolates were sensitive to the Gram-positive spectrum antibiotics and the β-lactam antibiotics. Resistance to vancomycin was similar to that of the previously reported LAB isolates.[214, 215] Antibiotic resistance can be triggered by a number of mechanisms, such as enzyme modifications and inactivation of the antibiotics mediated, cell wall impermeability, modifications of antibiotic binding molecules.[216] In addition, the corresponding genes of antibiotic resistance of *Bifidobacterium* did not transfer to the pathogenic organisms.[217] Antibiotic resistance appears to be intrinsic and the degree depends on the genus or species.[212]

Probiotics are useful in the management of pathogenic gut infections and prevent to antibiotic-related diseases, travel diarrhea and rotavirus. In our study 4 LAB isolates differently impacted the tested pathogens. The mostly probiotics could produce antimicrobial such as organic acids, bacteriocin, possibly one of the powerful mechanisms in fighting infectious diseases.[218] Similar results were obtained by Sandra Tejero-Sariñena et al. (2012)[219], who reported that antimicrobial activity of

Bifidobacterium against *Salmonella* typhimurium, *E. coli*, *Enterococcus faecalis*, *S. aureus* and *C. difficile* was mediated by organic acids. The ability of all isolates to inhibit pathogenic bacteria growth has been also attributed to synthesis of antibacterial, such as antibiotics or bacteriocins.[220] Additionally, ability to produce organic acids resulting in pH reduction could inhibit the growth of pathogens. The antibacterial activity generally correlated with the lowering pH effect.[221]

Analysis of 16S rRNA gene sequences confirmed the results from the cluster analysis and revealed that 4 LAB isolates were 100%, and 99% relatively similar to the genus *Lactobacillus* and *Bifidobacterium*. A phylogenetic tree was constructed based on their 16S rRNA gene sequences utilizing the neighboring method. The results showed that the MSMC36-9, MSMC37-3, and MSMC37-4 were similar to *Lactobacillus paracasei* JCM 1171^{T} while MSMC83 belonged to *Bifidobacterium animalis* subsp. *lactic* DSM 10140^{T}.

The *Bifidobacterium* spp. are considered generally recognized as safe (GRAS).[222] In addition, they are a core member of the human gastrointestinal microbiota and are dominant populations in most individual[223, 224], constituting > 1 % of the intestinal microbiota in adults[225] and representing up to 90% of microorganisms in the fecal of healthy breastfed infants.[226] Interestingly, they are among the first reported isolation of strictly anaerobic microorganisms colonizing the gut within the first week of life in full term neonates.[227] Zuo et al. (2015) reported that 13 out of approximately 200 colonies isolated from three feces samples of breast-fed infants were bifidobacteria consisting of *B. adolescentis*, *B. longum*,*B. breve*, *B. catenulatum*, and *B. bifidum*.[228] Moverover, *Bifidobacterium animalis* is a probiotic strain certified by the Food and Drug Administration of Thailand. Additionally, the strain *B. animalis* subsp. *lactis* is widely used in industrial production of fermented foods.[229] Moreover, this strain showed higher degree of stress resistance in the industry processes than other strains. [230]

Among 4 LAB isolates, *Bifidobacterium animalis* MSMC83 had the best antioxidant activity in chemical and cellular assays. In addition, it exhibited maximum

potential probiotic. Therefore, *B. animalis* MSMC83 was selected to use in the study of antioxidant activity *in vivo* and its application in fermented milk products.

Oxidative stress induced by D-galactose has 3 different pathways. In the first pathway, high level of D-gal lead to an increase in galactitol through a reaction catalyzed by galactose reductase. The accumulation of galactitol in cells affects osmotic pressure and causes abnormal of the antioxidant defense system. Additionally, D-galactose can stimulate ROS production through stimulation of NADPH oxidase. D-galactose is oxidized into aldehydes and hydrogen peroxide which can generate ROS. This pathway is an important factor in quickly accelerating the mechanisms that cause oxidative stress.[231] Previous studies have found that excessive amounts of ROS lead to oxidative stress which it has been confirmed to be a leading cause of many types of organ damage.[232] The liver produces large amounts of ROS, which lead to liver damage and its function is gradually declined.[233] Additionally, the liver is extremely responsible for detoxification, and most of the D-gal is metabolized in the liver.[234] Results of organ weight showed different liver weights among D-gal model, probiotic, and normal groups ($P < 0.05$). Body, liver and total fat weight of probiotic and ascorbic acid groups decreased, compared with D-gal model group ($P < 0.05$). The changes in the liver weight in the D-gal model group clearly indicated liver damage. Excessive ROS from intake of high dose of D-gal increase lipid oxidation and fat transport into cells, causing fat deposition. As an increase in fat accumulation occurs, the body's and total fat weight increase as well.[229, 235] Li B et al. (2018) showed that oral administration of *L. helveticus* KLDS1.870 alleviated the changes caused by D-gal, including reduced liver weight and liver damage.[236] Thus, administration of *B. animalis* MSMC83 had the potential abilities in repairing the D-gal induced liver oxidative stress damage.

Moreover, oxidative stress from long-term exposure to D-gal can cause inflammation and affect insulin resistance.[237] As the level of insulin resistance increases, the amount of glucose in the bloodstream increases, resulting in more free radical formation in the mitochondrial dysfunction.[238] Moreover, biochemical markers of liver oxidative stress, are the permeability of the cell membranes that affect the releasing

aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in the blood.[183] Our study found that *B. animalis* MSMC83 reduced fasting blood glucose and the level of serum AST in D-gal induced oxidative stress. While the ALT level in probiotic group tends to decrease. The results of Yu X et al., (2016) demonstrated that ALT and AST decreased significantly in *L. mucosae* LMU1001 groups, indicating their protective effect on liver.[184] Moreover, previous studies showed that hypertriglyceridemia and hypercholesterolemia are closely related to oxidative stress.[239] D-gal induced oxidative stress was also associated with significant lipid metabolism changes.[240] The level of triglyceride and total cholesterol in D-gal model group increased but decreased with administration of *B. animalis* MSMC83. Therefore, lowering-triglyceride and total cholesterol levels by receiving *B. animalis* MSMC83 may help reduce risk factors for atherosclerosis and cardiovascular complications. Moreover, *B. animalis* MSMC83 may decrease insulin resistance and protect the structure of the hepatocellular membrane in oxidative stress.[241]

Free radicals are eliminated through the body's redox system via generated antioxidant, which reduces the risk factors of oxidative stress.[242, 243] The antioxidant can be derived into 2 groups including antioxidant enzyme and antioxidant non-enzyme.[99] The superoxide dismutase (SOD), catalase, and glutathione peroxidase (GSH-Px) are primary antioxidant enzymes.[99] SOD is an important and common enzyme within cells to prevent oxidative damage through the catalytic process of superoxide ions to H_2O_2.[244, 245] Whereas catalase enzymes prevent the production of highly toxic hydroxyl radicals by accelerating the decomposition of hydrogen peroxide.[246] Additionally, GSH-Px is the key antioxidant enzyme in the glutathione (GSH) system that scavenges free radicals by accelerating the reduction of hydrogen peroxide to a non-toxic product and removing highly reactive lipid peroxide in cell membranes.[109, 247] As SOD, GSH-Px, and catalase levels increase, ROS levels can be reduced to prevent the oxidation of proteins, fats and DNA.[248] SOD, catalase, GSH-Px, and MDA activities were used to assess the protective effect of *B. animalis* MSMC83 in oxidative stress conditions. The results of this study showed that the activities of antioxidant enzymes in serum

increased and the oxidation product MDA activity decreased in serum by the treatment of *B. animalis* MSMC83 in the D-gal include oxidative stress. Remarkably, *B. animalis* MSMC83 has the same antioxidant capacity as ascorbic acid (vitamin C) by improvement in serum levels of SOD, catalase, GSH-Px, and MDA *in vivo*. The mechanism of antioxidant modulation is to produce antioxidant metabolites, increase levels of antioxidant metabolites of the host and regulate the intestinal microbiota.[123] Several probiotic can produce and secrete SOD.[170] According to the study by Kullisaar et al. (2002), two *L. fermentum* strains expressed generation of SOD in oxidative stress condition.[249] Moreover, probiotic was able to enhance antioxidant metabolites system of host.[250] Similar to our findings, the previous researches reported that oral administration of *L. plantarum* CCFM10 could raise SOD and GSH-Px activities and lowering the level of MDA in serum.[251] The study of Xu R. et al in 2011 reported that *B. animalis* RH significantly increased SOD, catalase, and total antioxidant capacity activities in D-galactose induced mouse models.[252] Similar to the previous study, *B. longum* BAMA-B05BAu-B1024 and *B. animalis* LPL-RH enhanced the SOD and GSH-PX activities and reduced the MDA activity in D-gal model mice.[253] The transcription factor NF-κB responded directly to oxidative stress because ROS mediates the activation of redox-sensitive transcription factor NF-κB.[254, 255] The extracellular polysaccharide LAB was able to inhibit NF-κB and ROS production to prevent LPS-induced inflammation in RAW 264.7 macrophages.[256] Similarly, *L. plantarum* FC255 treated mice expressed and translocated of liver Nrf2, promoting the transcription of genes antioxidant enzymes via Nrf2-Keap1-ARE pathway.[257] Moreover, LAB was able to downregulate ROS-producing enzymes in male rats.[258] Therefore, these results suggested that *B. animalis* MSMC83 increase intracellular antioxidant enzymes activities and reduce peroxidation product levels in oxidative stress.

The liver is the important organ of D-gal metabolism.[259] Oxidative stress causes liver inflammation, leading to secretion of pro-inflammatory cytokines such as tumour necrosis factor (TNF-α), interleukin-1 beta (IL-1β) and interleukin-6 (IL-6).[260] ROS activates the NF-κB transcription factor and regulates the expression of genes that

induce inflammation. The effect of this activation causes the release of pro-inflammatory cytokine and other substances that damage tissues and organs.[261] Thus, we first assessed the pro-inflammatory response to liver cytokines. The levels of TNF-α in serum were significantly increased in the D-galactose injected control group, while the levels of TNF-α of rats induced oxidative stress receiving *B. animalis* MSMC83 were significantly decreased. Previous studies have shown that *B. longum* and *B. animalis* alleviated pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6 in the hippocampus of D-gal model mice through inhibition of NF-κB / TLR4 signalling.[253] These findings indicated that *B. animalis* MSMC83 may protect liver inflammation against oxidative stress in a D-gal induced model.

An imbalance in the gut microbiota cause many health problems such as increase in oxidative stress[262], inflammation[263], and immune disorders.[264] The function of probiotics to modulate intestinal microbiota and intestinal disease has been studied.[265] Previous studies supported that oxidative stress induces the dysbiosis of gut microbiota.[266] The present study found that the amount of LAB and *Bifidobacterium* decreased and *E. coli* increased in the D-gal model compared to the control group. Interestingly, the administration of the *B. animalis* MSMC83 group resulted in gut microbiota constituents, similar to that of the control group. Regulation of the gut microbiota is also one mechanism of reducing ROS.[46] Abnormal gut microbiota, excessive pathogens, induces intestinal epithelial ROS generation.[140] LAB has the potential to regulate homeostasis of the gut microbiota via redox signaling.[267] These result is in agreement with the study by Zhao J et al. (2018), *L. plantarum* CCFM10 and *L. plantarum* RS15-3 changed the composition of the microbiota at the phylum and the genus levels after induced by D-gal to similar to normal group.[268] These findings suggested that administration of *B. animalis* MSMC83 protects against changes in gut microbiota, possibly one of the mechanisms for resistance to oxidative stress.

The pH profiles of the yoghurts fermented by regular yoghurt starter cultures as a control sample and yoghurt fermented by starter cultures combined with *B. animalis* MSMC83 were studied. The fermentation time of probiotic yoghurt was shorter than the

control sample. The difference in fermentation time may be attributed to *B. animalis* MSMC83 promoting the growth of starter cultures. The shorter fermentation time when co-fermented with probiotics was also found when *L. casei* was added.[269] Enhancement of acidification rate in probiotic yogurts, however, is strain dependent. Some probiotic strains such as, *L. rhamnosus*, *L. acidophilus*, and *B. lactis*, were inhibited when grown with yogurt starter cultures.[270] The viable counts of starter cultures and probiotic during storage are presented. During 4 weeks of storage, the bacterial counts were constant in control sample and were increased in the probiotic yoghurt.

In general, the food industry suggests a level of 10^6 CFU/g at the time of consumption for probiotic bacteria to have the expected health effects.[271] According to the results of present study, starter cultures and probiotics grew well and showed the stability of viable cells after storage. The major factors affecting the viability of probiotic depending on the strain, acidity, storage temperature, oxygen content and food matrix.[272, 273]

The average pH values ranged from 4.67 to 3.72 for probiotic yoghurt and from 4.49 to 4.23 for control yoghurt during the storage. The decrease in pH during storage is consistent with other studies Gueimonde et al. (2004) reported decreasing pH levels to between 3.9 and 4.2 during storage of 14 commercial yoghurts fermented with starter culture and probiotics.[274] The relation between decline in pH values and storage time may be due to the continuous fermentation of LAB in yoghurt during storage.[275] When the pH drops below 4.4, *Streptococcus* stopped growing while *Lactobacillus* and *Bifidobacterium* were still able to grow and produce amino acids, resulting in an decrease in the pH of the product.[276] Therefore, the residual acidification during storage can be attributed to the residual enzyme activity of microorganisms used in yoghurt production.

Firmness of the probiotic yoghurt added with *B. animalis* MSMC83, was significantly increased firmness ($P < 0.05$) during storage when compared with control yoghurt. According to Mani-López et al. (2014), firmness of fermented dairy products was strain dependent. Some LAB may produce mucogenic such as

exopolysaccharides, which enhanced the strength of protein matrix, resulting in improved firmness during storage [277, 278]

The syneresis values of both yoghurts significantly increased with storage time ($P < 0.05$). The addition of *B. animalis* MSMC83 in probiotic yoghurt significantly decreased the syneresis compared to the control yoghurt. The longer the fermentation time increases the time allowed for rearrangements of the gel network. This longer fermentation time, therefore, resulted in larger pore formation and increased syneresis.[279]

Syneresis is defined as separation of aqueous phase from continuous phase or gel network, which is a reversible indicator of the quality of yoghurt.[280] It may be spontaneous or may occur only when the gel is mechanically disrupted by cutting, freezing, or centrifugal force.[281] The increase in syneresis during storage may be described as post-acidification of yoghurts even they were stored at 4°C.[282] Similar results were observed when other probiotic strains, *L. plantarum* and *B. bifidum* were added to yoghurts.[283, 284]

The storage modulus (G') characterized the degree of elastic as solid-like and the loss modulus (G") characterized the degree of viscous as liquid-like properties.[285, 286] Our results showed an elastic characteristic in both yoghurts during storage. Fermentation time is the factor responsible for the differences in the moduli.[287] The rate of acidification directly affects the rheological properties, which is related to the different starter and incubation temperature and time. Increasing the rate of acidification resulted in gels with higher G' values.[288]

The storage modulus (G') values were higher than the loss modulus (G") values at frequency of 1 Hz in both yoghurts across the entire measuring range. In probiotic yoghurt, the maximum G' value was observed at 4th week and the lowest G' value was observed during initial week of storage. As compared to the control yoghurt, G' and G" of probiotic yoghurt were significantly higher ($P < 0.05$).

The change in viscosity in both yoghurts during 4 weeks of storage was not significant, although the viscosity values slightly increased. These results are in

agreement with the G' values because the development of apparent viscosity in yogurt is related to the aggregation of casein micelles and gel formation.[289]

Conclusions

Four LAB isolates, MSMC36-9, MSMC37-3, MSMC37-4 and MSMC83 showed antioxidant capacity including DPPH radical scavenging capacity, hydroxyl radical scavenging capacity in chemical assays and intracellular free radical level on Caco-2 cells. They exhibited potential probiotic characteristics including resistance to gastrointestinal conditions, and adhesion property on Caco-2 cells. Identification of 4 LAB isolates revealed that MSMC36-9, MSMC37-3, MSMC37-4 were assigned to *Lactobacillus paracasei* while MSMC83 was assigned to *Bifidobacterium animalis.*

Among 4 LAB isolates, *Bifidobacterium animalis* MSMC83 had strong antioxidant activity in chemical and cellular assays. In addition, it exhibited the highest probiotic potential. Therefore, *B. animalis* MSMC83 was selected to be analyzed for antioxidant activity in D-galactose-induced oxidative stress rats. The results of this study revealed that *B. animalis* MSMC83 was able to increase antioxidant enzymes that consist of superoxide dismutase, catalase, and glutathione peroxidase, and decrease malondialdehyde levels, the end product of lipid peroxidation. Moreover, administration of *B. animalis* MSMC83 prevented liver inflammation and modulated hemostasis gut microbiota.

B. animalis MSMC83 could be used in the production of probiotic yoghurt. The yoghurt fermented with starter cultures in combination with *B. animalis* MSMC83 required shorter fermentation time and showed superior physicochemical behaviors during cold storage. These results indicated that *B. animalis* MSMC83 is a potential probiotic strain for the production of yoghurts and other food products with increasing antioxidant capacity.

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