SCREENING OF LACTIC ACID BACTERIA THAT HAVE HEALTH PROMOTING EFFECT FROM THAI LOCAL FERMENTED FOODS

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การคัดกรองแบคทีเรียกรดแลคติกที่มีประโยชน์ต่อสุขภาพจากอาหารหมักพื้นบ้านไทย

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เอ็มดี ฟาริดันนาบิ นาเย็ม : การคัดกรองแบคทีเรียกรดแลคติกที่มีประโยชน์ต่อสุขภาพจากอาหารหมักพื้นบ้านไทย. (SCREENING OF LACTIC ACID BACTERIA THAT HAVE HEALTH PROMOTING EFFECT FROM THAI LOCAL FERMENTED FOODS) อ.ที่ปรึกษาหลัก : ณัฐธิดา โชติช่วง, อ.ที่ปรึกษาร่วม : ชาญชัย บุญหล้า

ความชราภาพเป็นหนึ่งในปัจจัยด้านสุขภาพที่สำคัญอย่างมาก โดยสาเหตุของความชราภาพมาจากอนุมูลอิสระออกซิเจนและการทำงานของอนุมูลอิสระดังกล่าว ซึ่งทำให้เซลล์ในร่างกายเกิดความเสื่อมถอย และยังเป็นปัจจัยหลักที่ทำให้เกิดโรคต่างๆ เช่น มะเร็ง, โรคหัวใจและหลอดเลือด, เบาหวานประเภทที่ 2, ความดันโลหิตสูง, การเสียสมดุลของชนิดจุลินทรีย์ที่สำคัญในระบบทางเดินอาหาร และรวมถึงโรคอื่นๆ โดยจุลินทรีย์กลุ่มโพรไบโอติกในอาหารหมักสามารถช่วยส่งเสริมประโยชน์เชิงสุขภาพด้านต่างๆ รวมถึงมีฤทธิ์ในการต้านอนุมูลอิสระและช่วยชะลอวัย ส่งผลให้โพรไบโอติกมีความเป็นไปได้ในการพัฒนาเป็นอาหารเสริม จากปัจจัยดังกล่าวจึงเป็นสิ่งที่น่าสนใจในการคัดกรองโพรไบโอติกโดยเฉพาะกลุ่มแบคทีเรียกรดแลคติก (LAB) ซึ่งเป็นกลุ่มแบคทีเรียที่รู้จักกันอย่างดี ที่มีฤทธิ์ต้านอนุมูลอิสระจากอาหารหมักพื้นบ้านไทย งานวิจัยนี้มีวัตถุประสงค์เพื่อคัดกรอง LAB ด้วยอาหารเลี้ยงเชื้อ MRS ที่มี $CaCO_3$ จากอาหารหมักพื้นบ้านไทยจากภูมิภาคต่างๆ (ภาคกลาง ภาคเหนือ ภาคตะวันตก และภาคตะวันออกเฉียงเหนือ) ทั้งอาหารหมักที่ได้จากพืชและจากเนื้อสัตว์จำนวน 31 ตัวอย่าง รวมถึงทำการวิเคราะห์คุณสมบัติการเป็นโพรไบโอติกและฤทธิ์การต้านอนุมูลอิสระ จากผลการทดลอง พบว่า จากตัวอย่างอาหารทั้งหมด พบ 18 ไอโซเลทที่น่าจะเป็น LAB เนื่องจากพบวงใสที่ชัดเจน บนวุ้นอาหาร MRS ที่มี $CaCO_3$ และไอโซเลทส่วนใหญ่แสดงฤทธิ์ในการกำจัดอนุมูลอิสระ DPPH ได้มากกว่า 70% ทั้งในอาหารเลี้ยงและตัวเซลล์แบคทีเรีย ยกเว้น 2 ไอโซเลทที่ได้จาก ปลาส้มและกุ้งจ่อม มีฤทธิ์ในการกำจัด DPPH ในตัวเซลล์ได้ 49.12 และ 58.08 % ตามลำดับ อย่างไรก็ตาม มีเพียงหนึ่งไอโซเลท NYC8 ที่ได้จากอาหารหมักจากพืชที่แสดงสมบัติการเป็นโพรไบโอติก ได้แก่ การไม่ชอบน้ำ, ความทนทานต่อกรดและน้ำดี, และฤทธิ์ต้านแบคทีเรียก่อโรค นอกจากนี้ NYC8 ยังแสดงความสามารถในการรีดักชั่นเทียบเท่า L-cysteine 195.91 μM และความสามารถ ในการยับยั้งการเกิดปฏิกิริยาออกซิเดชั่นของไขมัน ได้ 44.34% สำหรับตัวเซลล์และ 4.34% สำหรับอาหารเลี้ยงเหลว รวมถึงยังเป็นหนึ่งในไอโซเลทที่มีฤทธิ์ในการกำจัด DPPH สูงที่สุด และเมื่อทำการตรวจสอบลำดับเบสของไรโบโซม 16S พบว่ามีความคล้ายคลึง *Lactiplantibacillus plantarum* ATCC 14917^T (ACGZ01000098) และ *Lactiplantibacillus argentoratensis* 6365^T (CP032751) 100% จะเห็นได้ว่า ไอโซเลท NYC8 นี้ อาจสามารถนำไปศึกษาและพัฒนาเพิ่มเติมในอนาคตเพื่อเป็นอาหารเสริมที่ส่งเสริมการชะลอวัย

สาขาวิชา	วิทยาศาสตร์และเทคโนโลยีทางอาหาร	ลายมือชื่อนิสิต .....
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Aging has become one of the most important health considerations. Aging is caused by the generation of reactive oxygen species and the activity of free radicals which degenerate the cells in the human body. Aging is the prime factor for different types of diseases like cancer, cardiovascular problems, type II diabetics, hypertension, imbalance of important gut microbiota, and other diseases. Probiotics from fermented foods aid in health promoting functionalities as a function of antioxidant activity and promoting longevity which allows them to be a potential source of supplement. These factors open an interesting prospect of screening well known probiotics, especially lactic acid bacteria (LAB), which containing antioxidant activity from various Thai local fermented foods. This study aimed to screen targeted lactic acid bacteria (LAB) which is well known bacteria group, from 31 plant and animal based Thai local fermented foods in different regions (central, north, west, and north-east) by $CaCO_3$ containing MRS agar and analyzed its probiotic properties and antioxidant activities. It was found that all 18 isolates considered as potential LAB as it had shown clear zone on $CaCO_3$ containing MRS agar, and most isolates exhibited over 70% scavenging of DPPH in both supernatant and pellet except two isolates from plasom and kung jom having 49.12% for supernatant and 58.08% for pellet respectively. But only one isolate, called NYC8, from plant based fermented food showed significant potential probiotic properties, including hydrophobicity, acid-bile tolerance, and antimicrobial activity. Additionally, NYC8 isolate showed reducing ability equivalent to L-cysteine 195.91 µM along as well as lipid peroxidation inhibition capacity 44.34% for pellet and 4.34% for supernatant, and it was the one that had the one of the highest DPPH scavenging activities. After doing 16S rDNA sequencing, it was found that NYC8 isolate had 100% similarity with *Lactiplantibacillus plantarum* ATCC 14917^T (ACGZ01000098) and *Lactiplantibacillus argentoratensis* 6365^T (CP032751). This isolated strain might be further analyzed and developed as a potential longevity promoting supplement product.

Field of Study:	Food Science and Technology	Student's Signature .....
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		Co-advisor's Signature .....

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Introduction

1.1 Rationale

Aging is an unavoidable phenomenon in a life cycle which is associated with the generation of oxidative stress that leads to malfunctioning of different organs in the living organism (*Tan et al.,* 2018). Aging related oxidative stress is the result of uncontrolled reactive oxygen species (ROS) and reactive nitrogen species (RNS) production which includes hydroxyl radical ($OH^{\cdot}$), superoxide radical ($O_2^{\cdot -}$), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), peroxynitrite ($ONOO^-$), and nitric oxide radical ($NO^{\cdot}$) (Chung *et al.,* 2009; Lennicke *et al.,* 2015). ROS in the system can be generated by two sources: endogenous and exogenous source. Endogenous ROS can be formed during several oxygen metabolisms like oxidation of biomolecules (lipid, protein, and nucleic acid) inside of cell because of respiration system takes place in mitochondria. Exogenous ROS formation are associated with several factors such as ecological contamination, radioactivity, severe drugs, infection by bacteria, uncontrolled iron consumption, imbalance of gut microbiota etc. (Davali *et al.,* 2018, Vona *et al.,* 2021). Exceeding ROS level beyond the capacity of endogenous antioxidant defense system, proteins, nucleic acids, lipids and carbohydrates of cell and tissues become susceptible to damage (Daly *et al.,* 2004). Numerous enzymatic antioxidants which are generated through different organs including superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) as well as non-enzymatic antioxidants including ascorbic acid, α-tocopherol, β-carotene could heal oxidative stress damage. As an example, superoxide radical in the system is generated by nicotinamide adenine dinucleotide phosphate (NADPH) oxidation. Generated superoxide radical is minimized as H_2O_2 and water by the presence of SOD. Conversion of H_2O_2 into water and oxygen molecules further takes place by the influence of GPx or CAT. Thus, the ROS generation in the living organism is detoxified (Zhang *et al.,* 2017). Aging is associated with several health complications such as diabetes, Alzheimer's disease, Parkinson's disease, cancer etc. (Banji *et al.,* 2014). Many synthetic supplements like antioxidant supplements are available in the market in the name of β-carotene, vitamin A & E, butylhydroxy-nisole, butylhydroxytoluene and others to overcome the aging complications (Li *et al.,* 2018

and Lademann *et al.*, 2011). Probiotics from fermented foods having diverse functions, could be a great replacement of single functioned commercial supplements.

Fermentation is an ancient and economical method of processing and storing of food, that improves the shelf life of perishable fresh foods, maintains stability and safety of consumable foods, prevents microbial infestation, and enhances digestibility and mineral bioavailability (Nout, 2013; Tamang *et al*., 2016; Terefe, 2016). The functional properties of fermented foods are dependent on the salt concentration during fermentation and fermentation process. The evident effect of salt in fermented food is to suppress the growth of pathogens and regeneration of health promoting bacteria (Park *et al*., 2014). Additionally, these health-promoting microbes are considered as potential probiotics; some of them are scientifically proven health benefits include lowering the risk of type II diabetics, showing anti-aging and anti-obesity effect by their metabolic activities (Cui *et al*., 2015; Kim *et al*., 2011a; Das *et al*., 2020).

The general meaning of probiotic is “good for life”. Probiotics are defined according to WHO as “live microorganisms which administered in adequate amounts confer a health benefit on the host” (FAO UN & WHO, 2006). Probiotics are considered helpful against cancer, different allergic effects, diabetics, coronary heart disease, aging, lactose intolerance etc. They are also effective in treating stress, anxiety, depression and stimulating mood and cognitive activities (Sivamaruthi *et al*., 2019). Above 10^{14} CFU/ml microorganisms reside in human gastrointestinal tract, out of these *Lactobacilli and Bifidobacterium* are considered as conventional probiotics and have shown considerable antioxidant properties (Kim *et al*., 2020). Lactic acid bacteria (LAB) are one of the major sources of probiotics because of their availability in most consumable fermented foods. *Lactobacillus* spp is one of the most popular isolated genera of LAB where probiotics have been isolated. Besides this, other LABs are *Leuconostoc, Lactococcus, Pediococcus, Enterococcus, and Streptococcus* (Hill *et al*., 2014). In the past decades, *Lactobacillus* spp. are the most widely appreciated and used probiotics which have been used for treating human health complications (Singh *et al*., 2013). In general, *Lactobacillus* spp. are gram positive, catalase negative and non-spore forming (McCoy and Gilliland, 2007). This species had been isolated in

past decades most extensively considering its enhancement of antioxidant, anti-aging, and longevity (Woo *et al*., 2014). It was recorded that health promoting activities of probiotics had been found to be different in species to species, even different in same species because of difference in strains. *Lactobacillus helveticus* KLDS1.8701, *Lactobacillus fermentum* JX306, *Lactobacillus plantarum* FLPL05, *Lactobacillus mucosae* LMU1001, *Lactobacillus plantarum* LPL0902 & LPL0302 etc. are the potential candidates for enhancing of antioxidant activity in the animal model which was confirmed prior *in vitro* analysis of antioxidant properties like DPPH, H_2O_2 resistance and reducing ability measurement (Li *et al.,* 2018; Zhang *et al.,* 2020; and Yu *et al.,* 2016). On the other hand, strains like *Lactobacillus fermentum* MBC2 (Schifano *et al.,* 2019), *Lactobacillus fermentum* JDFM216 (Park *et al.,* 2017), *Lactobacillus fermentum* JX306 (Zhang *et al.,* 2020), *Lactobacillus rhamnosus* strain GG (ATCC 53103) (Yun *et al.,* 2022), *Lactobacillus fermentum* U-21 (Marsova *et al.,* 2020), *Lactobacillus gasseri* SBT2055 (Nakagawa *et al.,* 2016) etc. have been used for prolonging life span, growth, and healing liver, kidney, brain, spleen damages by promoting antioxidant activity *in vivo*. Besides this *Lactobacillus plantarum* HY7714 delays skin aging (Lee *et al.,* 2015). As it was reported that, numerous strains of *Lactobacillus* spp. had been testified to possess antiaging effects possibly for their free radical scavenging activity and promoting effect of antioxidant enzymes (Zhao *et al*., 2017). Most importantly food safety of LAB had been authorized as safe by conducting clinical trials (Saarela *et al*., 2000). Worldwide mostly LAB had been isolated from dairy based products and available in the market as an ingredient of dairy based products. Nowadays, a few non-dairy based LAB rich products are available in the global market like juice of apple, tomato, pineapple, and orange with *Lactobacillus sanfranciscensis* (Zhu *et al*., 2020); sohiong juice enriched with *Lactobacillus plantarum* (Vivek *et al*., 2020) and juice of pomegranate with *Lactobacillus plantarum* ATCC 14917 (Mantzourani *et al*., 2019). A few studies also reported the isolation of LAB from non-dairy products mustard (Lin *et al*., 2021), dry fermented sausage (Mora *et al*., 2015), and fermented vegetables (Xiao *et al*., 2020).

1.2 Objective

Considering the health benefits of lactic acid bacteria and availability of fermented in Thailand, this study was designed to:

- ✓ Isolate lactic acid bacteria from local Thai fermented foods.
- ✓ Analyze the potential probiotic properties and antioxidant activities of isolated strains *in vitro.*

1.3 Hypothesis

The local Thai fermented food would be the good source for isolation of lactic acid bacteria which had high antioxidant properties and probiotic properties.

1.4 Scope

This study focused on two main parts:

- ✓ The Isolation and screening of lactic acid bacteria from local Thai fermented foods
- ✓ Determination potential probiotic properties and antioxidant activities of isolated strains in vitro.

Review of Literature

2.1 Fermented food

Fermented is one of the most ancient processing of foods and beverages in different geographic locations of the world. Fermentation is known as the steady disintegration of complex organic molecules initiated by microorganisms or enzymes (FAO, 1998). In general, carbohydrate molecules are converted to alcohol or organic acid during fermentation. Fermentation process varies based on the tradition and cultural preference which leads to the generation of different fermented foods and beverages in different communities. The distinct process of fermented foods has been passed down to generations to hold cultural identity. Initially, fermentation process was adopted solely to survive in winter season during drought periods but later the unique flavor and texture of fermented foods and beverages also became a major consideration.

2.2 Classification of fermented foods

The diversity of fermented foods is associated with traditional morals, cultural acceptability, food habits, and sensory acceptance. So, a single classification of fermented foods isn't sufficient to represent the whole area of fermented foods. In this study, only a few classifications will be discussed to give an overview of fermented foods and beverages that are mostly consumed in different regions.

In general, fermented foods are categorized as animal based and plant based considering the source of raw materials (Muhialdin *et al.*, 2021). Animal based fermented foods are obtained from meat, fish, and dairy sectors. Fermented meat products are mostly consumed in USA, Europe, and North Africa with different processing measures (Chammem, Issaoui, De Almeida, & Delgado, 2018). Fermented meat products are associated with mostly LAB particularly *Lactobacillus* species that includes *L. alimentarius*, *L. pentosus*, *L. plantarum*, *L. versmoldensis* as well as *Pediococcus* species that includes *P. acidilactici* and *P.pentosaceus* (Tamang et al., 2016). Mostly consumed fermented meat products are in the form of fermented sausage, salami, and jerky which have been processed from mutton, beaf, and pork. *Basterma* is one kind of sausage which is prepared from minced beef and widely accepted in Middle east countries like Egypt, Iraq, Jordan, Lebanon, and Syria

(Zaccheo, Palmaccio, Venable, Locarnini-Sciaroni, & Parisi, 2017). *Nem chua* is another sort of ground fermented meats which is prepared from lean pork and is popular in Vietnam. Besides fermented meats, fermented fish products are widely popular in Asian countries like Thailand, Indonesia, Malaysia, Japan, Philippine, and others. Mostly consumed fermented fish products include fish sauce like *Budu* and normal fermented fish *belacan* or *kapi*. Fermented dairy products are another part of animal based fermented foods which have become a major consideration of people diet chart especially in North America, Europe, Middle East, and North Africa (Tamang *et al.,* 2020). The acceptability of fermented dairy products because of its health promoting compounds like peptides, amino acids, vitamins, and minerals (Santiago-L´opez *et al.,* 2018). There are some fermented dairy products, including yogurt and cheese, are famous in Asia as well with different names. LAB is mostly dominant in fermented dairy products, but yeast and mold has significant impact on the fermentation process. On the other hand, plant based fermented foods have a wide range of diversities and hold the major portion of the marketplace. Recently interest has been shifting towards plant based fermented foods in Asia because of their great history of health promoting activities, affordable price limit, and wide range of diversities. Health promoting activities of plant based fermented foods are associated with the presence of necessary microorganisms and bioactive compounds including amino acids, peptides, vitamins, and phenolic compounds. Apart from these factors, extreme demand for vegetarian and vegan diets influences the food industries towards innovations. Mostly consumed plant based fermented foods in Asia are fermented glutinous rice (*tapai pulut*), tapioca (*tapai ubi*), *dosa*, *kimchi*, *natto* (Ray, Ghosh, Singh, & Mondal, 2016; Merican & Quee-Lan, 2004), tempeh, and fermented vegetables: cabbage, mustard, olives, ginger, and others (Tamang *et al.,* 2016). Plant based fermented beverages: vinegar and *kombucha* (fermented sugared tea) have also got significant appreciation after covid pandemic because it can be used in treating several health complications (Villarreal-Soto *et al.,* 2019; Tan, Muhialdin, & Meor Hussin, 2020; Muhialdin et al., 2019).

African traditional food habit is composed of a significant portion of fermented foods including fermented maize, millet, cassava, meat, dairy products, and

alcoholic beverages (Tamang and Samuel, 2010). Fermented foods in Africa can be categorized in following groups: fermented non-alcoholic cereals, fermented starchy root crops, fermented vegetable protein, fermented animal protein, and alcoholic beverages based on the raw materials used during the fermentation process (Olasupo *et al.,* 2010). **Table 1** represents the diversities of fermented foods in Africa along with the associated microflora.

Table 1: *Fermented foods in Africa (Franz et al., 2014)*

Product	Area of production	Fermentable substrate	Microorganisms reported to be involved in the fermentation
African, non-alcoholic cereal-based foods			
Mahewu (magou)	South Africa	Maize, sorghum or millet	*L. delbrueckii* subsp. *bulgaricus; L. delbrueckii* subsp. *delbrueckii; Leuconostoc spp*.; heterofermentative *lactobacilli*
Ogi	Nigeria, Benin	Maize, sorghum or millet	Ped. pentosaceus, L. fermentum, L. plantarum, yeast (Saccharomyces cerevisiae, Candida kruseii)
Koko and Kenkey	Ghana	Maize, sorghum or millet	*W. confusa, L. fermentum, L. salivarius, L. vaccinostercus, L. pantheris, Pediococcus spp*. and yeast
Uji	East Africa	Maize, sorghum or millet	*L. plantarum, L. paracasei, L. fermentum, L. buchneri, Ped. acidilactici, Ped. pentosaceus*
Kisra	Sudan	Sorghum	LAB
Hussuwa	Sudan	Sorghum	*L. fermentum, Ped. acidilactici, Ent. faecium* (minor proportions)
Injera	Ethiopia	Sorghum	*Candida guillermondii*
Ting	Botswana, South Africa	Sorghum	*L. fermentum, L. plantarum, L. rhamnosus*
Obusera	Uganda	Millet	LAB
Mawe	Benin	Maize	*Lact. lactis, Ped. pentosaceus, L. plantarum*
Kunu-zaki	Nigeria	Millet, sorghum	*L. fermentum, P. pentosaceus, W. confusa, Ent. Faecalis*
Bogobe	Botswana	Sorghum	Unknown
Potopoto	Congo	Maize	*L. gasseri, L. plantarum/paraplantarum, L. acidophilus, L. delbrueckii, L. reuteri, L. casei, Bacillus spp., Enterococcus spp.*
Dégué	Burkina Faso	Millet	*L. gasseri, L. fermentum, L. brevis, L. casei, Enterococcus spp.*
Ben saalga	Burkina Faso	Millet	*L. plantarum* and other LAB

Table 1: Fermented foods in Africa (Franz *et al.*, 2014) (continue)

Product	Area of production	Fermentable substrate	Microorganisms reported to be involved in the fermentation
African fermented starchy root products			
Gari	West Africa	Cassava	L. *plantarum, L. fallax, L. fermentum (predominating) W. paramesenteroides, L. brevis, Leuc. pseudomesenteroides (minor proportions), Strep. lactis, Geotrichum candidum, Corynebacterium manihot* (also reported)
Lafun	Nigeria	Cassava	*L. fermentum, L. plantarum, W. confusa, yeast (Saccharomyces cerevisiae, Pichia scutulata, Klyveromyces marxianus, Hanseniaspora guillermondii),* and *Bacillus spp.*
Fufu	NIgeria	Cassava	*Ped. pentosaceus, L. fermentum, L. plantarum*
Kivunde	Tanzania	Cassava	*L. plantarum*, other LAB, yeast
Chikawngue	Zaire	Cassava	LAB, yeast
Cingwada	East and Central Africa	Cassava	Unknown
Kocho	Ethiopia	Ensette or Abyssinian banana (Ensette ventricosum)	LAB yeast
Agbelima	Ghana	cassava	*L. plantarum, L. brevis, L. fermentum, Leuc. mesenteroides, also Bacillus spp., Candida tropicalis, Geotrichum candidum, Penicillium spp.*
African fermented animal proteins			
Nono (milk curd)	Northern part of West Africa	Milk	LAB
Maziwalala	East Africa	Milk	*"Strep." (Lact.) lactis, Strep. thermophilus*
Leban (sour milk)	Morocco	Milk	*Lactic streptococci (lactococci), Leuc. lactis, Leuc. mesenteroides subsp. cremoris*
Wara	West Africa	Milk	*Lactococcus lactis, Lactobacillus spp.*
Ergo	Ethiopia	Milk	*Lactobacillus spp., Lactococcus spp.*
Kulenaoto	Kenya	Milk	*L. plantarum, L. fermentum, L. paracasei, L. acidophilus, also lactococci, leuconostocs and enterococci*
Sethemi	South Africa	Milk	*Lactobacilli, lactococci, yeast (Debaromyces hansenii, Saccharomyces cerevisiae, Cryptococcus curvatus)*
Guedj	Senegal	Fish	Lact. lactis
Bonome (stink fish)	Ghana	Fish	Unknown

Cereal based fermented foods are widely consumed fermented foods in Africa as cereal is the staple food which supports around 80% of total calories. These foods are the major source of dietary protein which makes these foods both complementary diet for infants and staple diet for adults. Apart from this, fermented leafy vegetables in Africa are widely consumed because of their natural and easy manufacturing process along with their anti-nutrient reduction capacity. Root crops like cassava is another popular crop all over Africa which has been fermented to produce wide varieties of fermented products and extend the shelf life. On the other hand, most fermented animal proteins come from dairy based products. Fermented dairy products are consumed in the form of fermented sour milk, fermented butter, traditional ghee, and fat free sour milk (Gonfa *et al.*, 2001). Major microflora that are associated with this fermentation process are LAB with a concentration of 10^7 to 10^8 CFU/ml along with some types of yeasts (Mathara *et al.*, 2004).

Thailand is a land of fermented foods where fermented foods are mostly consumed compared to any other Asian countries. Traditional Thai fermented foods can be classified in two groups: salted and non-salted fermented foods. Salted fermented foods include food sources from animal, fish, soyabean, fruits, and vegetables where salt concentration ranges from 0.5–20% (**Table 2**). Salted fish products are prepared either with the addition of different carbohydrates or without the addition of carbohydrates based on the local practices. Some fermented fish products are prepared with higher concentration of salt including *Budu, Kapi, Nam pla* and others. Carbohydrate rich salted fermented fish products contain cooked, boiled, fermented, and roasted rice as a food source for microorganisms associated with the fermentation as well as improve the sensory qualities of the fermented foods. Some fishery salted fermented foods are prepared with fruits e.g., *Pla mam* is prepared by fish and pineapple fermentation. Apart from fermented fish products, animal based fermented products have significant contribution to Thai people diet. Meat is often fermented with different fruits and vegetables to reduce the cost and impart distinct flavor. In general, fermented meat products are fermented as sausage (*Sai-Krok-Prieo*) or minced form with banana leaf wrapper (*Naem*). Beside this, salted fermented fruit products are also popular among the Thai young generations.

Fermented fruit and vegetable products are mostly preferred because of their easy preparation process, flavor, and shelf life. These products are consumed in different styles based on the locality e.g., *Miang* (fermented tea leaves) with fruits like peanut, roasted coconut, ginger, and garlic; fermented green mango with syrups; and *Nam-Prik* (chilli paste) with other foods.

***Table 2**: Salted Thai traditional fermented food (Yongsmith & Malaphan, 2016)*

Product name	Thai name	Typical characteristics	% Salt	Associated microflora
		Fishery products		
		Fish		
Bu-Du	บูดู	Muslim sauce, fish sauce	19.4–20.6	*Bacillus laterosporus, Bacillus subtilis*
Jing-Jang	จิงจัง	Fermented fish	13.2–24.8	*Pediococcus halophilus*
Ka-Pi-Pla	กะปิปลา	Fermented fish paste	10.9–19.8	*Bacillus licheniformis, Bacillus sphaericus*
Nam-Pla	น้ำปลา	Fish sauce	22.8–26.2	*Bacillus pantothenticus, Bacillus sp., Halobacterium salinarum*
Pla-Chao	ปลาเจ่า	Thai sweetened fermented fish	5.0–6.4	*Bacillus sp.,Endomycopsis sp.*
Pla-Jorm	ปลาจ่อม	Fermented fish	2.6–10.2	*Micrococcus sp., Pediococcus halophilus, Saccharomyces cerevisiae, Staphylococcus pidermidis*
Pla-Paeng-Daeng	ปลาแป้งแดง	Red fermented fish	2.3–4.0	*Bacillus polymyxa, Bacillus subtilis*
Pla-Raa	ปลาร้า	Fermented fish	11.5–23.9	*Bacillus licheniformis, Bacillus subtilis*
Pla-Som	ปลาส้ม	Fermented fish	4.0–10.7	*Bacillus sp., Candida sp. Lactobacillus brevis, Lactobacillus plantarum*
Som-Fak	ส้มฟัก	Thai fermented fish cake	3.6–3.9	*Bacillus sp., Candida sp., Lactobacillus brevis, Lactobacillus fermentum, Lactobacillus plantarum*
Som-Khai-Pla	ส้มไข่ปลา	Fermented fish egg	0.9–4.1	*Lactobacillus sp*
Som sai pla	ไตปลา	Fermented fish viscera	13.5–25.3	*Bacillus polymyxa, Bacillus subtilis*
		Shrimp		
Ka-Pi	กะปิ	Fermented shrimp paste	14.0–40.1	*Bacillus sp., Bacillus licheniformis, Bacillus sphaericus*

Product name	Thai name	Typical characteristics	% Salt	Associated microflora
Koong-Jorm	กุ้งจ่อม	Fermented shrimp	3.2–9.4	*Micrococcus sp., Pediococcus halophilus, Saccharomyces cerevisiae, Staphylococcus epidermidis*
Koong-Som	กุ้งส้ม	Fermented shrimp	7.5–10	*Bacillus sp., Candida sp., Lactobacillus casei var. casei, Lactobacillus casei var. alactosus Lactobacillus plantarum*
		Others		
Hoi-Ma-Laeng-poo-Dorng	หอยแมลงภู่ดอง	Fermented sea mussel	11.4–12.5	*Pediococcus halophilus, Staphylococcus aureus, Staphylococcus epidermidis, Trichosporon sp.*
Hoi-Som	หอยส้ม	Fermented shell	3.5–4.4	*Pediococcus halophilus*
Nam-Poo	น้ำปู	Fermented crab paste	10.3	
Poo-Khem	ปูเค็ม	Fermented crab	7.8–17.3	
		Animal products		
Mam	มั่ม	Fermented beef or pork sausage	2.3–7.1	*Micrococcus sp., Staphylococcus epidermidis,*
Naang	หนาง	Fermented pork or beef	1.2–3.4	*Bacillus sp., Lactobacillus sp*
Naem	แหนม	Fermented pork/beef	2.5–2.8	*Lactobacillus acidophilus, Lactobacillus brevis, Lactobacillus cellobiose, Lactobacillus plantarum*
Sai-Krok-Prieo	ไส้กรอกเปรี้ยว	Fermented sausage	1.1–1.9	*Debaryomyces sp., Lactobacillus salivarius, Lactobacillus paracasei, Lactobacillus plantarum*
Som-Dteen-Wooa	ส้มตีนวัว	Fermented ox hoof and hock	little	
Som-Neua	ส้มเนื้อ	Fermented beef	3.1	
		Fruit products		
Buay-Dorng	บ๊วยดอง	Fermented Japanese apricots	18.3	

Product name	Thai name	Typical characteristics	% Salt	Associated microflora
Kra-Thorn-Dorng	กระท้อนดอง	Pickled santol	0.5–1.7	
Loog-Jan-Ted-Dorng	ลูกจันทร์เทศดอง	Pickled nutmeg fruits	0.2–0.6	
Loog-Pling-Dorng	ลูกปลิงดอง	Pickled bilimbi	4.1	
Loog-Tor-Dorng	ลูกท้อดอง	Fermented peach	23	
Lum-Pee-Dorng	หลุมพีดอง	Fermented Lumpee	1.0–1.8	*Lactobacillus sp., Pichia sp.*
Ma-Dan-Dorng	มะดันดอง	Pickled garcinia	0.4–2.7	
Ma-Kaam-Dorng	มะขามดอง	Fermented tamarind	2.4–2.5	
Ma-Kaam-Pom-Dorng	มะขามป้อมดอง	Fermented Indian gooseberry	1.2–1.6	
		Fruit products		
Ma-Kork-Nam-Dorng	มะกอกน้ำดอง	Pickled Spanish plums	3.7–4.2	
Ma-Muang-Dorng	มะม่วงดอง	Fermented green mango	1.7–11.3	
Ma-Nao-Dorng	มะนาวดอง	Pickled lime	12.4–18.6	
Ma-Pring-Chae-Im	มะปริงแช่อิ่ม	Sweetened plum mango	1.3	
Ma-Yom-Dorng	มะยมดอง	Pickled star gooseberry	0.9–9.7	
Poot-Sa-Dorng	พุทราดอง	Pickled jujube	0.7–2.5	*Bacillus sp., Lactobacillus plantarum*
Sa-Mor-Dorng	สมอดอง	Fermented olive	1.2	*Candida sp.*
Too-Rian-Prieo	ทุเรียนเปรี้ยว	Fermented durian	7.1	*Candida sp., Lactobacillus sp*
		Vegetable products		
Ton-Horm-Dorng	ต้นหอมดอง	Fermented spring shallots	1.5–2.3	*Candida sp., Lactobacillus plantarum*
Horm-Dorng	หอมดอง	Pickled shallots	2.0	
Hooa-Pak-Kaat-Dorng	หัวผักกาดดอง	Dried salted Chinese radish	11.9–13.2	*Debaryomyces sp.,*
Ka-Lam-Dorng	กระหล่ำดอง	Pickled cabbage	1.7	

Product name	Thai name	Typical characteristics	% Salt	Associated microflora
Khing-Dorng	ขิงดอง	Pickled ginger	4.0–5.3	
Kra-Tiam-Dorng	กระเทียมดอง	Pickled garlic bulbs	3.2–5.0	
Lam-Peuak-Dorng	ลำเผือกดอง	Fermented taro stalk	3.0	
Loog-Kra-Dorng	ลูกกระดอง	Fermented perah seeds	0.8	*Lactobacillus plantarum*
Loog-Niang-Dorng	ลูกเนียงดอง	Fermented djenkol bean	2.7	*Bacillus polymyxa,*
Loog-Riang-Dorng	ลูกเรียงดอง	Fermented parkia seeds	1.7–3.4	*Bacillus sp., Candida sp., Lactobacillus plantarum*
Ma-Keua-Dorng	มะเขือดอง	Pickled eggplant	2.6–3.8	
Ma-Keua-Proh-Dorng	มะเขือเปราะดอง	Fermented garden eggplants		
Miang	เมี่ยง	Fermented tea leaf, pickled tea leaf	0.1–1.5	
Nor-Mai-Dorng	หน่อไม้ดอง	Fermented bamboo shoot	0.5–6.4	*Bacillus cereus, Bacillus sp., Lactobacillus plantarum*
Pak-Kaat-Dorng	ผักกาดดอง	Pickled mustard greens	1.6–7.3	*Lactobacillus brevis, Lactobacillus plantarum*
Pak-Kaat-Dorng-Haeng	ผักกาดดองแห้ง	Dried fermented mustard greens	39.8	*Debaryomyces sp., Lactobacillus brevis*
		Vegetables Product		
Pak-Koom-Dorng	ผักกุ่มดอง	Pickled crataeva	1.5–2.6	
Pak-Naam-Dorng	ผักหนามดอง	Pickled lasia	0.7–2.3	*Lactobacillus plantarum,*
Pak-Sian-Dorng	ผักเสี้ยนดอง	Fermented wild spider flower or leaf tips	0.8–2.1	*Bacillus sp., Lactobacillus plantarum*
Sa-Tor-Dorng	สะตอดอง	Fermented sator seed	1.3–3.7	*Lactobacillus plantarum, Lactobacillus sp*
Tang-Chai	ตั้งฉ่าย	Dried fermented cabbage	15.8	
Tooa-Li-Song-Dorng	ถั่วลิสงดอง	Fermented peanut sprouts	2.1	
Tooa-Ngork-Dorng	ถั่วงอกดอง	Pickled mungbean sprouts	1.1	

Product name	Thai name	Typical characteristics	% Salt	Associated microflora
		Soybean products		
See-Iu	ซีอิ๊ว	Soy sauce	19.2–21.7	*Aspergillus oryzae, Aspergillus sojae, Bacillus sp.*
Tao-Hoo-Yee	เต้าหู้ยี้	Fermented soybean cheese	12.6–19.6	*Actinomucor elegans,*
Tao-Jieo	เต้าเจี้ยว	Soybean paste	15.7–19.0	*Aspergillus oryzae, Lactobacillus delbrueckii*

Note: High salted products, 15.5–20 % or above; medium salted products, 4.5–15 %; and low salted products, 0.5–4 %

Non-salted Thai fermented food products (**Table 3**) are generally prepared from rice, fruits, cassava, and soyabean. This group can be classified into two categories: Non-alcoholic and alcoholic products. Non-alcoholic fermented products are mostly consumed as fermented rice noodles (*Ka-Nom-Jeen*), fermented red rice (*Khao-Daeng*), dessert (*Ka-nom-Thuai-Fu*) from flour with coconut cream, roasted soyabean (*Thua-Nao*) and others. Alcoholic Thai fermented products mainly prepared from three sources: rice, fruits, and cassava. Under this category, fermented products preparation requires unique preparation devices, and their drinking materials are different e.g., rice wine is consumed by long bamboo straw (Yongsmith & Malaphan, 2016).

Table 3: *Non-salted Thai fermented foods (Yongsmith & Malaphan, 2016)*

Product types	Thai names	Product names	Associated microflora
		Alcoholics	
From rice	ลูกแป้ง	Loog-Paeng	*Issatchenkia orientalis Endomycopsis fi buligera*
	ข้าวหมาก	Khao-Maak	*Amylomyces rouxii, Aspergillus sp., Chlamydornucor sp., Endomycopsis sp.*
	น้ำขาว	Nam-Khao	*Amylomyces rouxii*
	อุ	Ou	
From fruits	ไวน์	Wine	*Saccharomyces cerevisiae*
	น้ำตาลเมา	Nam-Dtaan-Mao	
From cassava	ตา-แป	Taa-Pae	

Product types	Thai names	Product names	Associated microflora
		Non-alcoholics	
From rice	อั้งคัก	Ang-Kak	
	ขนมจีน	Ka-Nom-Jeen	*Bacillus subtilis, Lactobacillus casei var. casei*
	ขนมฝักบัว	Ka-Nom-Fak-Booa	
	ขนมถ้วยฟู	Ka-Nom-Thuai-Fu	
	ขนมตาล	Ka-Nom-Dtaan	
From beans	ถั่วเน่า	Thua-Nao	*Bacillus amyloliquefaciens Candida sp., Klebsiella pneumoniae*
From coconut water	วุ้นมะพร้าว	Woon-Ma-Prao	
From pineapple	วุ้นสับปะรด	Woon-Sap-Paroat	

2.3 Microorganisms associated with fermented foods

Fermentation is a spontaneous process where food products are simplified with the involvement of several microorganisms including LAB, other groups of bacteria, yeast, mold, and surprisingly some pathogens. LAB has the major impact on almost all food and beverage fermentation processes (Soemarie, Milanda, and Barliana, 2021). The associated bacteria have the potential to produce organic acids which allow to increase the shelf life of fermented products. Moreover, these bacteria are naturally found in nature as well as different parts of the human body including intestine, mouth, skin, mucosa membranes, and genitals which indicates the probable positive health impact of these natural bacteria on human body. Considering the impacts of LAB, these bacteria have been used in many applications that include pharmaceuticals, generation of probiotics, initiation of fermentation process for both food and feed, and handling of biological agents (Harzallah and Belhadj, 2013). Bacteria that are associated with fermentation process, can be classified into two categories: phylum Firmicutes and Actinobacteria. Phylum Firmicutes is composed of many genuses including *Lactobacillus, Lactococcus, Leuconostoc, Oenococcus, Pediococcus, Enterococcus, Tetragenococcus, Aerococcus, Streptococcus, Carnobacterium, Weissella, Alloiococcus, Symbiobacterium,* and *Vagococcus.*

Phylum Actinobacteria comprises of only two genuses: *Atopobium* and *Bifidobacterium*. Most species under Firmicutes phylum are in LAB group that are associated with salt based fermented foods e.g., *Bacillus subtilis, Lactobacillus fermentum, Lactobacillus plantarum etc.* are prominent in fermented fish having high salt concentrations. There are a few species which can be found in non-salted fermented foods as well e.g., *Lactobacillus casei, Bacillus amyloliquefaciens, klebsiella pneumoniae* etc. are associated with fermented rice. LAB can be found in both in plant, vegetable, dairy and meat sources. But LAB have been isolated normally from dairy sources as they are rich in carbohydrate (lactose) and of low pH which facilitate the LAB growth and enhance lactic acid production. LAB generation in fermented foods is related to several factors including fermentation time, fermentation temperature, type of fermentation, salt concentration, pH, packaging type etc. All the factors are interlinked with each other and show different impact on the growth of LAB after combination. Salt concentration reduces the growth of LAB at the initial fermentation state because of the quick metabolic cycle which make LAB less acid and salt tolerance. Over the fermentation time LAB become homo-fermentative (only producing lactic acid) which make it stable at low acid levels and high salt concentration (Chorianopoulos *et al.*, 2005; McFeeters and Pérez-Díaz, 2010). Concentrations of LAB (except acid tolerance strains e.g., *Lactobacillus acidophilus*) increases up to certain fermentation time and later the concentration starts decreasing because of generation of acid in the fermentation medium (Lin and Chien, 2007). Fermentation under room temperature enhances the growth of LAB compared to the low temperature (Zhou, Drouin and Lafrenière). Fermentation conditions (aerobic and anaerobic) also plays a significant role on the survival of LAB. Anaerobic condition initially facilitates the growth of LAB (especially probiotic strains) by maintaining the optimum pH but generation of other microflora over the time minimize the growth of LAB by generating lactic acid, acetic acid, and other LAB inhibitory metabolites (Smetanková *et al.*, 2012). Finally, raw materials and their constituents have a significant impact on the availability of LAB. Fermented foods that contain cellulose, hemicellulose, protein etc. compounds, go through complex fermentation. Normally, LAB cannot utilize hemicellulose, cellulose, and inorganic nitrogen compounds directly and it requires several pretreatments

(Svetlitchnyi et al., 2022; Yan and Zhang, 2019). Improper pretreatment before fermentation results in generation of phenolic aldehydes which reduce the growth of LAB (Qiu *et al.*, 2019). Initially, there might be a few numbers of LAB generate but growth of other competitive groups and lack of substrate for fermentation resist the growth of LAB.

2.4 Probiotics

Fermented foods are associated with a group of microorganisms which initiates the fermentation process. Additionally, some groups of microorganisms generate during the fermentation process which shows some health stimulating effects. The group of microorganisms that are associated with health stimulating functions are considered probiotics. According to WHO and FAO probiotics are defined as, "Live microorganisms which when administered in adequate amounts, confer a health benefit on the host". In general, many microorganisms show significant health benefits, but not every microorganism is considered probiotic. A microorganism is considered probiotic under certain conditions (**Figure 2.1**). Probiotic strain must pass through gastrointestinal tract with considerable concentration of living cell and provide benefits to the gut section. Briefly, a probiotic strain must tolerate the acidic environment in stomach in presence of gastric juice, bile tolerance at intestine, attach to the epithelial tissue of intestinal mucosa, interact efficiently with gastrointestinal cell components, provide immune functions, provide antimicrobial effect towards pathogenic strains (*Staphylococcus aureus*, *Escherichia coli*, *Listeria monocytogenes*, *Salmonella* spp. etc.), and able to stimulate and modulate the growth of gut microflora (Mattila-Sandholm *et al.*, 2002; Millette *et al.*, 2008; O'May and MacFarlane, 2005; Parvez *et al.*, 2006). Probiotic strains are mostly the genus *Lactobacillus* and *Bifidobacterium*. The *Lactobacillus* genus is the most important one because it comes from most consumable fermented food products.

LAB is the major groups of probiotics which are associated with food and feed fermentation. The LAB group contains mostly genus *Lactobacillus* along with other genera including *Enterococcus*, *Lactococcus*, *Oenococcus*, *Pediococcus*, *Streptococcus*, and *Leuconostoc* (Kandler and Weiss, 1986). Strains from *Lactobacillus* are rod shaped, gram positive, catalase negative, non-spore forming,

and hydrogen peroxide resistant. It is found in a wide range of foods that include animals, plants, and raw milk (Hammes and Vogel, 1995) which give this group a diversity of metabolites. The favorable environment for *Lactobacillus* growth is mostly anaerobic but it has considerable amount of growth in facultative anaerobic condition. The genus *Lactobacillus* prefers to grow in acidic conditions with pH range of 5.5-6.5 (Śliżewska, and Chlebicz-Wójcik, 2020) as they produce mostly lactic acid at the end of fermentation. *Lactobacillus* genus can be further classified into three categories based on their pathway of sugar fermentation: obligately homofermentative, organic substance converts into only lactic acid; facultatively heterofermentative, carbohydrate converts into lactic acid and acetic acid at the end of pathway; and heterofermentative, organic substance converts into acetic acid and CO_2 beside lactic acid (Kandler, 1983). Considering the composition and effectiveness of *Lactobacillus*, it has been used in fermentation of dairy products, fish, sausage, vegetables, and in silage preparation.

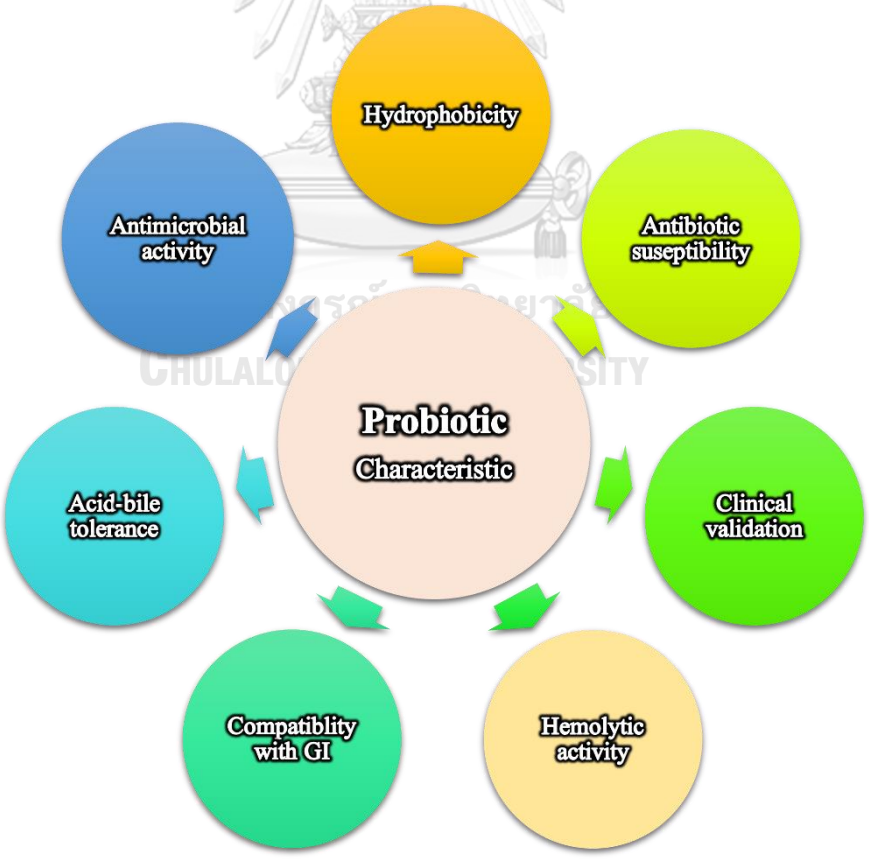

Figure 2. 1 Major characteristics of probiotics

2.5 Mechanism of probiotics in human body

2.5.1 Immune response and antiviral mechanisms of probiotics

Probiotics, by definition perform specific health promoting activities to this host. Health promoting activities of probiotics (**Table 4**) in living organisms are associated with several factors such as food selection, alteration of gut microflora, synthesis of bioactive molecules and others as shown in **Figure 2.2**. Probiotics are ingested in the human body by means of fermented foods (Marco *et al.,* 2021). Fermented foods have differences in nutrients' level from the raw food because of microbial interaction during fermentation process (Giraffa, 2004). During fermentation, monosaccharides and disaccharides level in fermented foods are reduced by different catabolic pathways which make fermented foods more acceptable and tolerable for human consumption (Nyyssölä *et al.,* 2020).

Fermented foods having high levels of polyphenol, convert into different phenolic compounds by the interaction of lactobacilli which enhance the bioavailability of compounds including flavonoids, tannins, and others (Septembre-Malaterre, Remize, and Poucheret, 2018). Probiotics in the fermented foods survive in digestion tract and accumulates in intestine. The accumulation of probiotics aids in synthesis of bioactive compounds, inhibition of pathogenic microflora (Parvez *et al.,* 2006), and influence the gut microflora (Li *et al.,* 2018). The major influencing bacteria that stimulate the gut microflora are probiotic LAB which has been claimed in different literature (Alm *et al.,* 2002). Gut microflora along with metabolites (bacteriocins, peptides, fatty acids, amino acids etc.) that generate from fermentation have a significant impact on the immune system of human (Zhang *et al.,* 2016). In LAB group, *Lactobacilli* is one of the major genera which improve immune response in human body by different mechanisms including higher generation of superoxide anion along with elevating the phagocytosis functioning and amount of plasma lysozyme (Harikrishnan, Balasundaram, & Heo, 2010). The improved immune response by probiotics is directly linked to the antiviral function of probiotics (Hardy, Harris, Lyon, Beal, & Foey, 2013). Bacteriocin from probiotic LAB was found to have a great impact on reduction of some specific viral multiplication (Cavicchioli *et*

al., 2018). Beside this, probiotics attach with intestinal lumen which minimize the attractions of viruses towards intestinal mucosa and protect it from infection (Karst, 2016).

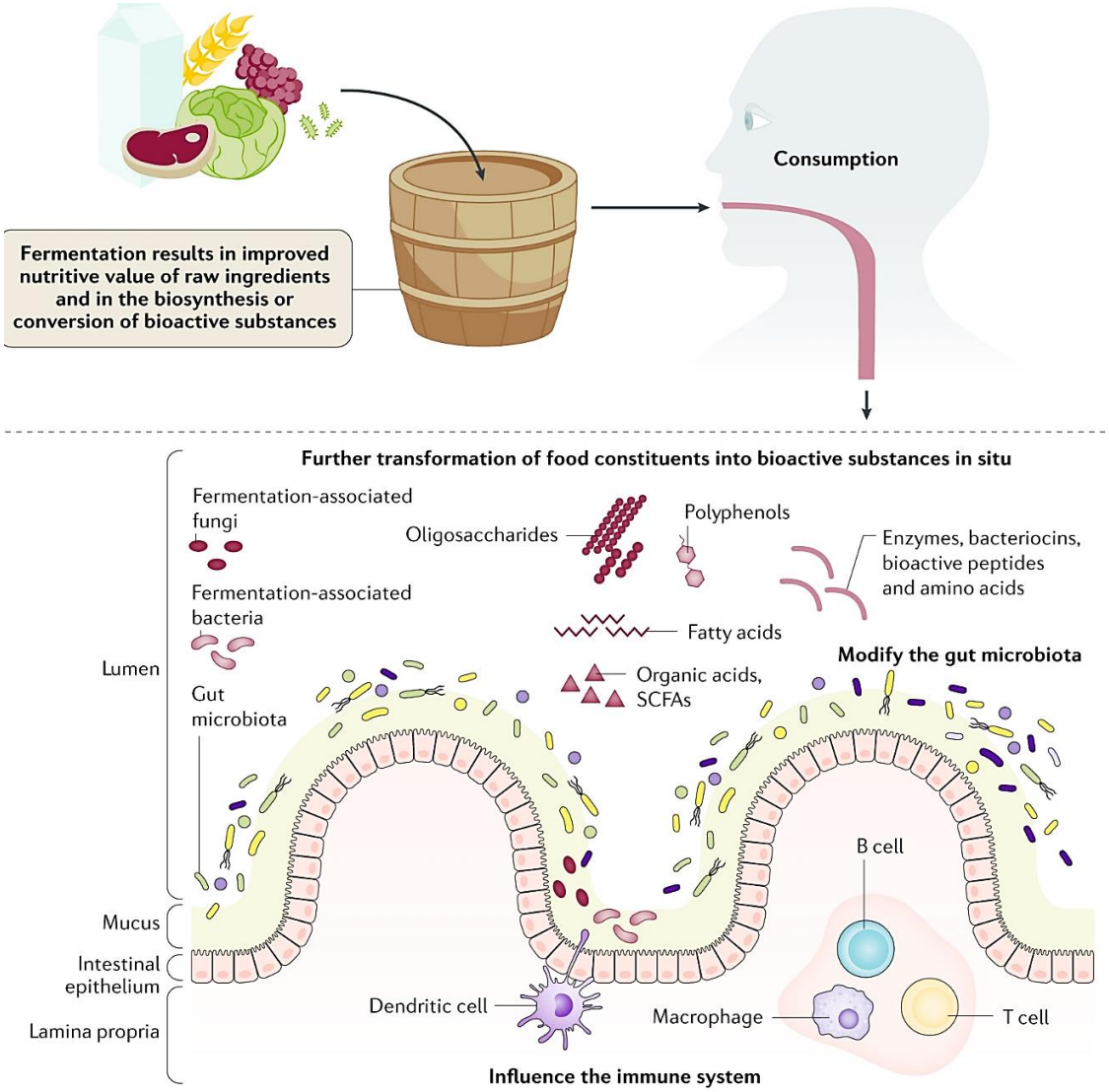

Figure 2. 2 Immune response functioning of probiotics (Marco et al., 2021)

2.5.2 Antioxidation mechanisms of probiotics

Antioxidant activity of probiotics is an important characteristic that scavenge or inhibit the free radicals in the human body and help to enhance health promoting effects. There are many mechanisms of probiotics that assist in antioxidant activity are: chelation of metal ion, enzymatic defense system, generation of antioxidant

metabolites, modulates signaling pathway, stimulating gut microbiota, and regulation of ROS producing enzymes (Wang *et al.,* 2017). Probiotics show chelation of metal ions (Fe^{2+} or Cu^{2+}) because of the presence of natural chelators in probiotics (Lin and Yen, 1999). Natural chelators in probiotics minimize the enzyme activity that initiates phosphate ester displacement and produce alkoxyl and peroxyl radical because of hydroperoxides decomposition (Halliwell *et al.,* 1995). Lin and Yen (1999) reported the chelating ability of the LAB, and they reported the maximum Fe^{2+} ion chelation in *Lactobacillus casei* KCTC 3260. Probiotics have their own enzymatic defense system to get rid of free radicals in the system. Major enzymatic defense systems in probiotics is assisted by SOD which is generated as Fe-SOD and Mn-SOD. SOD minimizes oxidative stress by breaking down the superoxide into hydrogen peroxide and water molecules (Landis and Tower, 2009). CAT is another enzyme that assist in enzymatic defense system by breaking down the hydrogen peroxide molecule into water which reduces the hydroxyl radicle in the system (Ho *et al.,* 2004). Mostly, LAB are CAT negative, however some strains have been reported as CAT positive. It was reported that *Lactobacillus casei* BL23 strain showed CAT producing ability which reduced the ROS level in the system to treat intestinal disorders (de Moreno de Leblanc *et al.,* 2004). Generation of different metabolites (glutathione, butyrate, and folate) by probiotics also assists the antioxidant activity. Glutathione containing probiotic strains are highly antioxidative and they have the potential to ROS in the system (Zilmer *et al.,* 2005). Folate is one type of vitamin which assists in DNA replication, repairing and methylation and its deficiency causes oxidative stress increment in type-2 diabetes (Al-Maskari *et al.,* 2012). There are many pathways that are mediated by probiotics to perform health promoting activities as a function of antioxidant activity. In general, antioxidant signaling pathways for probiotics are: Nrf2-Keap1-ARE (Koba *et al.,* 2009, Jones *et al.,* 2015), NFκB (Schreck, Rieber, and Baeuerle, 1991), Mitogen-activated protein kinases (MAPKs) (Seth *et al.,* 2008), and Protein kinase C (PKC) pathways (Blumberg, 1991). Additionally, probiotics reduce the ROS producing enzymes (NADPH oxidase, NOX; Cyclo-oxygenase, COX) to reduce the ROS generation in the animal body system. The reduction of NOX and COX by probiotics had been reported in many previous studies (Gómez-Guzmán *et al.,* 2015).

2.5.3 Antiaging mechanisms of probiotics

Aging is a natural, universal, and progressive deterioration of functional process that ultimately leads to death (Tan *et al.,* 2018). Theoretically, aging is the consequence of the oxidative stress that generates in response to ROS (**Figure 2.3).** Aging induces some health complications especially mitochondrial disfunction which leads to transformed electron transport system, reduction of mitochondrial fluidity, energy disproportion, and ultimately the failure of mitochondria (Eckmann *et al.,* 2013; Chistiakov *et al.,* 2014). ROS in the system is generated because of imbalance in antioxidant and prooxidant in the system (Zuo *et al.,* 2015). Beside ROS, reactive nitrogen species (RNS) has a significant impact on the generation of oxidative stress. Aging related reactive species are superoxide (O_2^-), hydroxyl radical (•OH), peroxynitrite (ONOO−), hydrogen peroxide (H_2O_2), reactive lipid aldehydes,and reactive nitric oxide (NO) (Chung *et al.,* 2009; Lennicke *et al.,* 2015). It has been found that there are nine hall marks of aging which can be further subdivided in three groups are: primary (genomic volatility, length of telomere, epigenetic modification, and proteostasis loss); antagonistic (uncontrolled nutrient sensing, dysfunction of mitochondria, and cellular senescence); and integrative hallmarks (collapse of stem cell and modified intercellular network) (Lo´pez-Otı´n *et al.,* 2013). The hallmarks of aging process has been significantly controlled by the gut microbiota which assists in maintaining health of human (Ding *et al.,* 2019) by controlling homeostasis (El Aidy *et al.,* 2012), intestinal functioning (Hayes *et al.,* 2018) along with development and functioning of brain (Sampson & Mazmanian, 2016). Gut microbiota alteration by probiotics influences Nrf-2 signaling pathway which acts as defensive protein against oxidative stress. Nrf-2 stimulates defense systems by binding with ARE sequence to promote the genes coding enzymes' transcription which act as scavengers against ROS as antioxidant defense system or detoxifying proteins to delay aging process (Chen *et al.,* 2016). Additionally, probiotics enhances antiaging potential by the activation of adenosine monophosphate activated protein kinase (AMPK) pathway (Hor *et al.,* 2019), TIR-1- JNK-DAF16 pathway (Zhao *et al.,* 2017), DAF-16/insulin-like signaling pathway (Grompone et al., 2012), as well as stimulating nuclear hormone receptor (Mangelsdorf *et al.,* 1995; Park *et al.,* 2018).

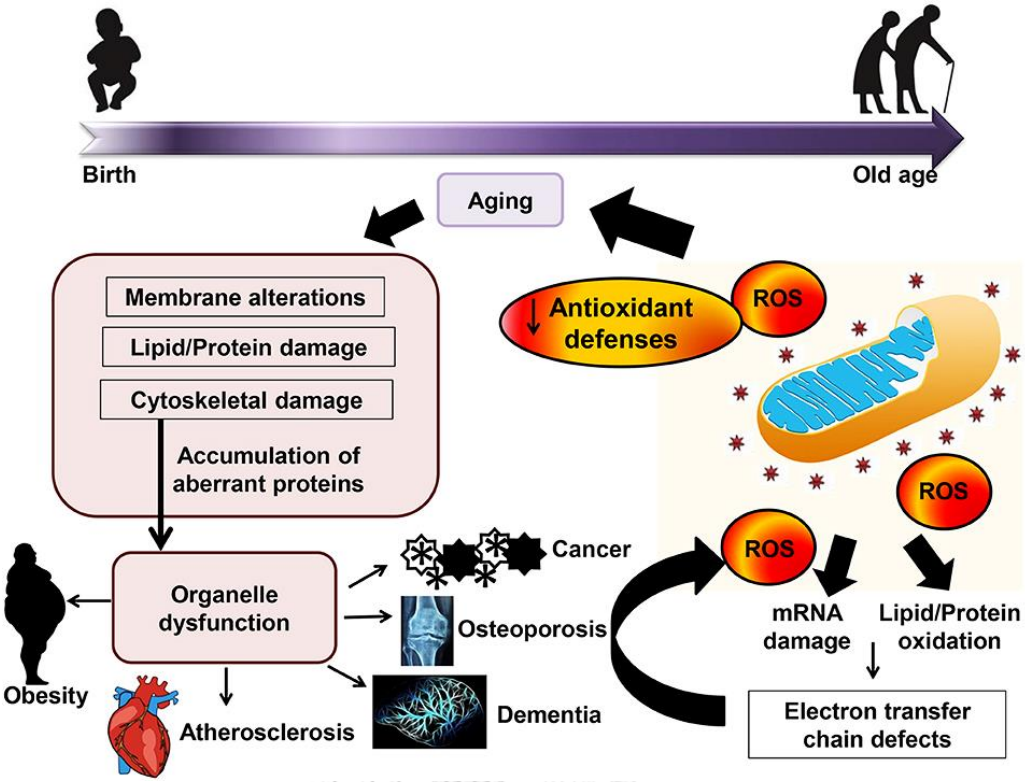

Figure 2. 3 Aging process and related disease as a function of oxidative stress (Tan et al., 2018)

Table 4: Source and health benefits of probiotics (Zhao et al., 2019)

Food	Country	Ingredients	Microorganisms	Health / Nutrition Benefits
(a). Based on different regions of milk fermented foods				
Acidophilus milk	Various countries	cow milk	Lactobacillus acidophilus,Lactobacillus. deLactobacillus rueckii subsp.bulgaricus,Lactobacillus. helveticus,Lactobacillus. paracasei subsp.paracasei,Lactobacillus. paracasei subsp.pseudoplantarum	intestinal disordersincluding chronicconstipation, diarrhea,colitis, sprue
Amasi or mukaka wakora or zifa	Zimbabwe	milk	Lactococcus. lactis subsp. lacti,Lactococcus. lactis subsp. lactisbiovar diacetylactis,Lactobacillus. paracasei subsp.paracasei,Lactobacillus. plantarum,Lactobacillus acidophilus,Leuconostoc. Mesenteroides subsp.mesenteroides,Enterococcus. faeciumEnterococcus. faecalis	inhibition of pathogens
Ayib	Ethiopia	goat milk	Lactobacillus sp,Lactococcus sp.	easy to absorb nutrients
Butter milk (various localnames)	Various countries	cow milk	Lactobacillus. bulgaricus,Lactococcus. lactis subsp. lactis,Lactococcus. lactis subsp. cremoris,Lactococcus. lactis subsp. lactis biovardiacetylactis,Leuconostoc. mesenteroide s subsp.cremoris	stimulates the power of digestion, treating hemorrhoids, IBS and other abdominal disorders.
Caplis	Japan	cow milk	Lactobacillus. helveticus,Saccharomyces cerevisiae	the source of proteins, fat, vitamins and minerals.
Cheese (various local names)	Various countries	cow milk, or goat milk, or sheep milk	Lactococcus. lactis subsp. lactis,Lactococcus. lactis subsp. cremoris,Sterptococcus Thermophilus,Lactobacillus. helveticus,Lactobacillus. deLactobacillus rueckii subsp.bulgaricus, yeasts, molds	it creates a buffer against the high acidic environment

Food	Country	Ingredients	Microorganisms	Health / Nutrition Benefits
Dahi (Indian yogurt)	India	cow milk or buffalo milk	Lactococcus. lactis subsp. cremoris,Lactococcus. lactis subsp. lactis,Sterptococcus Thermophilus,Lactobacillus. deLactobacillus rueckii subsp.bulgaricusLactobacillus acidophilus,Lactobacillus. helveticus,Lactobacillus. fermentum,Lactobacillus. casei,Lactobacillus. plantarum	intestinal disorders (dyspepsia, dysentery, diarrhea, etc); improved digestion, antibacterial effects in gut
Ergo or irgo	Ethiopia	cow milk	Lactobacillus sp.Streptococcus sp.	increased nutritional value
Katyk	Kazakhstan	cow milk	Sterptococcus Thermophilus,Lactococcus. lactis subsp. lactisbiovar diacetylactis,Lactobacillus. helveticus,Lactobacillus. deLactobacillus ruecki subsp.bulgaricus	increased nutritional value, easy to absorb nutrients
Kefir	Russia	cow milk or goat milk or sheep milk	Lactobacillus sp,Leuconostoc sp,Lactococcus sp.yeasts	antitumour properties; digestive system disorders
Kehran or karan or heran or lapte-akru	Siberia, Rumania	cow milk	Sterptococcus Thermophilus	increased nutritional value
Kishk or kisk, or kushuk (various localnames)	Egypt, Syria, Lebenon, (many other countries)	milk (yogurt)and wheat	Lactobacillus. casei,Lactobacillus. plantarum,Lactobacillus. brevis,Bacillus subtilis,Bacillus. lichenformis,Bacillus. megatherium,yeasts	increased nutritionalquality
Koumiss or kumys or coomys	Russia	mare milk or cow milk	Lactobacillus. De Lactobacillus rueckii subsp.bulgaricus,Lactobacillus acidophilusTorula yeast	hypolipidemic and immunomodulatory effects
Liban	Lebanon	cow, sheep, goat, or camel milk	Sterptococcus Thermophilus,Leuconostoc. lactis,Lactobacillus acidophilus,Kluyveromyces fragilis,Saccharomyces cerevisiae	potential ability to help with allergies and autism

Food	Country	Ingredients	Microorganisms	Health / Nutrition Benefits
Laban rayeb or leban rayeb, or raib	Egypt	cow or sheep milk	Lactococcus. lactis subsp. lactis, Staphylococcus. kefir, Lactobacillus. casei, Staphylococcus. faecalis	help probiotics colonize
Leban zeer	Egypt	cow milk	Lactobacillus. casei, Lactobacillus. plantarum, Lactobacillus. brevis	promote digestion
Leben (orLactobacillus en)	Zimbabwe, Morocco	cow milk	Lactic acid bacteria, Lactococcus. lactic subsp. lactis, Lactococcus. lactis subsp. lactisbiovar diacetylactis, Leuconostoc. lactis, Leuconostoc. mesenteroides subsp. Cremoris Leuconostoc. mesenteroides subsp. dextranicum, Lactobacillus sp.,yeasts, molds	reduce blood fat, fight cancer
Liban	Iraq	cow milk		reduce cholesterol
Lyubitelskii	USSR	cow milk	Sterptococcus Thermophilus, Lactococcus. lactis subsp. lactisbiovar diacetylactis	preventing cardiovascular disease in the elderly
Miziwa lalaor mala	Kenya	cow milk	Lactococcus. lactis subsp. cremoris, Lactococcus. lactis subsp. lactisbiovar diacetylactis, Leuconostoc. mesenteroides subsp.cremoris	improve human immune function
Nono	West Africa	cow, sheepmilk	Lactobacillus acidophilus, Lactococcus. lactis subsp. cremoris, Lactobacillus. deLactobacillus rueckii subsp.bulgaricus, Lactobacillus. helveticus, Lactobacillus. Plantarum, Lactobacillus. lactis subsp. cremoris	overcoming lactose intolerance
Nordic or Scandinaviansour milk (various localnames	Norway, Sweden	cow milk	Lactic acid bacteria, Lactococcus. lactis subsp. lactis, Lactococcus. lactis subsp. cremoris, Lactococcus. lactis subsp. lactisbiovar diacetylactis, Leuconostoc. mesenteroides subsp. cremoris	enhance flavor, prevention and treatment of diabetes and liver disease also have a certain effect
Skyr	Iceland	cow milk	Sterptococcus Thermophilus, Lactobacillus. Lactobacillus rueckii subsp. bulgaricus, Lactobacillus. helveticus	skyr is high in protein and calcium, and low in sugar and fat
Sour milk	Egypt	cow milk	Lactococcus. lactis subsp. lactis, Staphylococcus. kefir, Staphylococcus. cittrovorus, Lactobacillus. casei, Lactobacillus. plantarum, Lactobacillus. brevis, micrococci, coliforms	Promote gastrointestinal digestion
Susa	Kenya	camel milk	Lactobacillus acidophilus, Kluyveromyces fragilis, Saccharomyces cerevisiae	benefits for heart and immune health

Table 4: Continue

(b) Based on different regions of legume and cereals fermented foods

Food	Country	Ingredients	Microorganisms	Health/Nutrition Benefits
Bhallae	India	black gram	Lactic acid bacteria, yeasts	high protein, easy to absorb
Dawadawa	Nigeria	locust beans (Parkiabiglobosa) seeds	Bacillus sp.	antioxidants
Doenjang	Korea	soybeans	Aeromonas. oryzae	prevention of chronic diseases
Hama-natto	Japan	soybeans	Aeromonas. oryzae, Streptococcus sp., Pediococcus sp.	strong cytotoxic activity against cancer cells
Iru	West Africa	locust bean (Parkiabiglobosa) seed	Bacillus sp.	rich in B vitamins
Kecap	Indonesia	soybeans	Aeromonas. oryzae	antiobesity potential
Kenima	India, Nepal	soybeans		helps cholesterol esterification and protects fat-soluble vitamin E from damage
Kinema	India, Nepal	soybeans	Bacillus subtilus, Escherichia. faecium, Candida parapsilosis, Geotrichum candidum	increased nutritional quality
Meitauza	China,Taiwan	soybeans	Actinomucor elegans	reduce blood cholesterol, anti-mutagenic
Meju	Korea	soybeans	Aeromonas. oryzae, Rhizopus sp.	antiobesity potential
Miso	Japan	soybeans	Aeromonas. oryzae, Torulopsis etchellsii, Lactobacillus sp.	enhances the lingering aftertaste of cooked dishes
Natto	Japan	soybeans	Bacillus (subtilis) natto	possible stimulation of immune system
Ogiri-igbo	Nigeria	castor beans (Ricinus communis)	Aeromonas. oryzae,Rhizopus sp.	increased digestibility

Food	Country	Ingredients	Microorganisms	Health/Nutrition Benefits
(c) Based on different regions of vegetable fermented foods				
Dhamuoi	Vietnam	cabbage, various vegetables	Ln. mesenteroides, Lactobacillus. plantarums	removal of antinutrient compounds
Kawal	North Africa	vegetables	Lactobacillus. plantarum	enhancing food quality and safety
Kimchi	Korea	Korean cabbage, radish, various vegetables	Ln. mesenteroides, Lactobacillus. brevis, Lactobacillus. plantarum	Antimicrobial properties, antitumour acitivity, prevent constipation
Sauerkraut	Germany	cabbage	Ln. mesenteroides, Lactobacillus. brevis, Lactobacillus. plantarum, P. cerevisiae	source of vitamins and fibre
Tsukemono	Japan	Vegetables, rice flour	Lactic acid bacteria	improve the digestive functions, enhance the immune system,
Iru	West Africa	locust bean (Parkiabiglobosa) seed	Bacillus sp.	reduce the risk of colorectal cancer, control the serum cholesterol levels
(d) Based on different regions of fish and meat fermented foods				
Anchovy	Mediterranean countries, Argentina	anchovy	Lactic acid bacteria, yeasts	improve liver function and immunity
Burong dalag	Philippines	fish (dalag), rice	Ln. mesenteroides, P. cerevisiae, Lactobacillus. plantarum, S. faecalis, Micrococcus sp.	inhibit the proliferation of harmful bacteria
Burong hipon	Philippines	shrimp		inhibit the proliferation of harmful bacteria
Nahm	Thailand	pork, rice	Pediococcus sp., P. cerevisiae,	provide vitamins
Narezushi	Japan	fish, millet	Ln. mesenteroides,Lactobacillus. plantarum	prevent constipation and lower cholesterol
Nem-chua	Vietnam	pork, rice	Pediococcus sp,Lactobacillus sp.	prevent constipation and lower cholesterol
Guedj	Senegal	Fish	Lact. lactis	improve liver function and immunity

2.6 Screening of potential probiotic *Lactobacillus spp.* from fermented foods

The LAB group contains mostly genus *Lactobacillus* along with other genera including *Enterococcus*, *Lactococcus*, *Oenococcus*, *Pediococcus*, *Streptococcus*, and *Leuconostoc* (Kandler and Weiss, 1986). *Lactobacillus* genus is found in most consumable fermented foods including fermented milk, fruits, and vegetables (Hammes and Vogel, 1995). *Lactobacillus* genus particularly prefers to grow in acidic condition that is found in de Man, Rogosa and Sharpe (MRS) agar because of its composition. So, MRS agar is used as both culturing medium and primary screening of *Lactobacillus* from different fermented foods (De Man, Rogosa, and Sharpe, 1960). Recently, different concentrations of $CaCO_3$ has been used along with MRS agar to screen *Lactobacillus* as it makes clear zone near the colony because of the reaction between produced acid and $CaCO_3$ (Wu *et al.,* 2012). Aween *et al.,* (2012) reported the screening of *Lactobacillus acidophilus* strains from honey by using MRS agar containing 0.8% $CaCO_3$. Wu *et al.,* (2012) also reported the use of $CaCO_3$ (0.3%) with MRS agar for the screening of *Lactobacillus plantarum* AF1 from salted fish. Further screening of potential *Lactobacillus* is done by gram staining method (Tripathi and Sapra, 2020). *Lactobacillus* strains are gram positive, and rod shaped, so strains having rod shaped and gram positive are selected for the next analysis.

Probiotic properties of *Lactobacillus* strains are initiated by acid-bile tolerance to measure its survival potential in gastrointestinal tract. Artificial gastric (pH 1.8-2.5) and bile condition (pH 6.5-6.8) is generated by different chemicals and measured the survival potential by enumeration of *Lactobacillus* colonies and compare initial concentrations with final concentrations (Gbassi *et al.,* 2009; Liong and Shah, 2005; Khullar *et al.,* 2022). The attraction of *Lactobacillus* strains with intestinal epithelial tissue can be identified by cell surface hydrophobicity analysis of a strain (Khullar *et al.,* 2022). Hydrophobicity can be determined using different organic solvents including xylene, chloroform, and ethyl acetate. Xylene has an advantage over the other two solvents as it is free from electrostatic interactions, but chloroform acts as electron donor and ethyl acetate acts as electron acceptor which might affect the result of hydrophobicity (Martins *et al.,* 2009). Antimicrobial activity of *Lactobacillus* strains can be analyzed against pathogens including *Staphylococcus aureus* ATCC

25923, *Escherichia coli* ATCC 25922, *Clostridium difficile* DSM 1296, *Salmonella Typhimurium* ATCC1331 and others. Antimicrobial activity is mostly determined by two methods: spot on lawn and agar well diffusion method where diameter of the clear zone around the strain indicates antimicrobial activity (Khullar et al., 2022 and Pan *et al.,* 2009).

Table 5 Primer sequence

Primer	Primer sequence	bp	Ref
Forward	F-341 (5′-CCTACGGGAGGCAGCAG-3′)	450	Ritchie *et al.,* 2008
Reverse	786-R (5′- GACTACCAGGGTATCTAATC-3′)		
Forward	27F (5′-AGAGTTTGATCCTGGCTCAG-3′)	1500	El Oirdi *et al.,* 2021
Reverse	1492R (5′-ACGGTTACCTTGTTACGACTT-3′)		
Forward	27F (5′-AGAGTTTGATCCTGGCTCAG-3′)	2000	Yi *et al.,* 2019
Reverse	1495R (50-CTA CGG CTA CCTTGT TAC GA-30)		
Forward	F-341 (5′-CCTACGGGAGGCAGCAG-3′)	450	Ritchie *et al.,* 2010
Reverse	786-R (5′- GACTACCAGGGTATCTAATC-3′)		
Forward	GG (5′- CAATCTGAATGAACAGTTGTC-3′)	470	Brandt and Alatossava, 2003
Reverse	GG (5′- TATCTTGACCAAACTTGACG-3′)		
Forward	E-97800 (5′- ACGGGAACAGCCAATCAG-3′)	389	Brandt and Alatossava, 2003
Reverse	E-97800 (5′- GGCGTAGCTGGATTGTCTC-3′)		
Forward	Lc705 (5′- GATCGAGCGATACAACGC-3′)	241	Brandt and Alatossava, 2003
Reverse	Lc705 (5′- TGGGAGGATCATACGTGC-3′)		
Forward	p30F (5′-GTGATCGCAGTTGGAAAACTG-3′)	179	Galanis *et al.,* 2015
Reverse	p30R (5′-GTGATCGCAGGGAGATTATC-3′)		
Forward	p29AF (5′-GGGTAACGCCACAAGAAGC-3′)	401	Galanis *et al.,* 2015
Reverse	p29R (5′-GGGTAACGCCTTGCACTTTG-3′)		
Forward	LAcF (5′-GGAAGCTCAAGACCAAATCATG-3′)	397	Sheu *et al.,* 2009
Reverse	LAcR (5′-C TTCTTCAAAACATAAACTTGTG-3′)		

Identification of *Lactobacillus* strains is done mostly by 16s rDNA coding region sequence and PCR amplification. DNA extraction can be done considering many protocols including DNA extraction kit, boiling method, Kimchi modified method,

heat shock method, phenol-chloroform method, and others (Abdulamir *et al.,* 2010; Dashti *et al.,* 2009). DNA amplification of *Lactobacillus* strain in PCR is done using different primers such as in **Table 5** for targeting specific band. PCR amplified data is sequenced and compared to identify the potential *Lactobacillus* strain.

2.7 Analyzing antioxidant activity of potential probiotic *Lactobacillus spp.*

In vitro antioxidant activity of *Lactobacillus* strain can be determined by several means such as reducing ability, ABTS free radical scavenging activity (Li *et al.,* 2018), DPPH free radical scavenging activity, hydroxyl radical activity, superoxide anion radical scavenging activity, chelating activity of Fe^{2+} or $Cu^{2+,}$ lipid peroxidation inhibition capacity, quantification of enzymes (SOD, GPx), and metabolites identification (Tang *et al.,* 2017). DPPH and ABTS free radical scavenging activity, both are the most common protocols for analyzing antioxidant property. ABTS protocol is considered more precise (Floegel *et al.,* 2011), but DPPH is more commonly used because of the simplicity of the protocol. Many literatures analyzed the antioxidant activity considering 0.2 mM DPPH solution for *Lactobacillus* strains. Mostly, DPPH scavenging activity is represented equivalent to Trolox or gallic acid (Fei *et al.,* 2017; de la Fuente et al., 2021; and Zhou *et al*., 2020) but it can be represented as percentage scavenging capacity (Tang *et al.,* 2017; Li *et al.,* 2018). Antioxidant activity can be expressed by another property which is reducing ability. Normally, 2,4,6-Tripyridyl-S-triazine (TPTZ) (Bhat *et al.,* 2015) and potassium ferricyanide (Tang *et al.,* 2017) are used for the analysis of reducing ability. Reducing ability is expressed as the equivalent of BHT (butylated hydroxytoluene) standard or L-cysteine hydrochloride standard. Besides reducing ability, lipid peroxidation inhibition is a common mechanism that takes place in the system. There are several reasons for lipid peroxidation, but generation of free radicals is one of the major reasons for lipid peroxidation which can be terminated by the presence of antioxidants. Normally, lipid peroxidation inhibition capacity is determined by using linoleic acid as a lipid source (Tamg *et al.,* 2017). Besides linoleic acid, there are some other fatty acids can be used as a lipid source like liposomes (Virtanen *et al.,* 2007), egg yolk (DÜz, DoĞan, and DoĞan) etc. In general, lipid peroxidation capacity is expressed in percentage.

Materials and Methods

3.1 Chemical and apparatus

Chemical reagents

Analytical grade chemicals were used for the study.

- MRS agar (HIMEDIA, India)
- MRS broth (HIMEDIA, India)
- Peptone (HIMEDIA, India)
- Calcium carbonate ($CaCO_3$) (KEMAUS, Australlia)
- Sodium chloride (NaCl) (Qrec Chemicals, New Zealand)
- Phosphate buffer saline (PBS)
- Crystal violet (Qrec Chemicals, New Zealand)
- Iodine (Sigma-Aldrich, Germany)
- Ethanol (RCI Labscan, Thailand)
- Safranin (Azer Scientific Inc, USA)
- Pepsin (Sigma-Aldrich, Germany)
- Hydrochloric acid (HCl) (Qrec Chemicals, New Zealand)
- Sodium Hydroxide (NaOH) (Ajax Finechem co. Ltd., New Zealand)
- Bile salt (Sigma-Aldrich, Germany)
- Pancreatic enzyme (Sigma-Aldrich, Germany)
- Lipase (Sigma-Aldrich, Germany)
- 2,2-diphenyl-1-picrylhydrazy (DPPH) (Alfa Aesar, USA)
- Potassium ferricyanide (KEMAUS, Australlia)
- L-cysteine (HIMEDIA, India)
- Ferric chloride ($FeCl_3$) (Qrec Chemicals, New Zealand)
- Trichloroacetic acid (TCA) (Carlo Erba, France)
- Thiobarbituric acid (Carlo Erba, France)
- Tween 20 (RPI, USA)
- Ferus sulphate ($FeSO_4$) (Qrec Chemicals, New Zealand)
- Linoleic acid (Sigma-Aldrich, USA)
- Primers (vivantis, Malaysia)

- Buffer (vivantis, Malaysia)
- Magnesium chloride (vivantis, Malaysia)
- dNTP (vivantis, Malaysia)
- Agarose (HIMEDIA, India)

Apparatus

- Biosafety cabinet (Telstar, Bioultra, USA)
- Centrifuge (Hettich Universal 320 R, Germany)
- Microplate reader (Biochrome, 502182, England)
- Autoclave (Tomy, SX-500, Japan)
- Incubator (memmert, Germany)
- Hot air oven (Heraeus, Germany)
- Freezer (Panasonic, Japan)
- Refrigerator (Mitsubishi, Japan)
- Microscope (Nikon, YS100, Japan)
- pH meter (Mettler Toledo, Australia)
- Water bath (memmert, Germany)
- PCR device (BIO RAD, T100 Thermal Cycler, USA)
- Gel electrophoresis (Hercuvan, TT-HES-1, UK)
- Electronic balance (2 digit) (Sartorius model BP 310s, Göttingen, Germany)
- Electronic balance (4 digit) (Sartorius model BSA 2245, Göttingen, Germany)

3.2 Sample collection

Fermented foods were categorized into two groups: plant and animal based fermented foods. These foods were collected from different provinces of Thailand from local market. **Table 6** represented the selected fermented foods (Phak kard dong, Bai miang, Nam hed, Nho mai dong, Phak sian dong, Ton hom dong, Kao mak, pra ta pean, Naem, Plasom, Mum, Pla kem, Pla ra, Som sai pla) and their collection sources. The collected foods were used initially for isolation of potential lactic acid bacteria and stored in refrigerator for short time at 4^{o}C in case of necessity.

3.3 Isolation of lactic acid bacteria from Thai local fermented foods

Isolation of lactic acid bacteria was done considering Bautista-Gallego *et al.*, 2013 method with some modifications. Briefly, solid food samples (10 g) were homogenized in 0.1% peptone (20 ml) for uniform distribution. Then, took 1 ml from homogenized food sample and mixed with 9 ml peptone and again homogenized it. It was serially diluted 5 times with 0.1% peptone and then spread on MRS agar. For liquid sample, 1 ml of homogenized food sample was taken and serially diluted 5 times with 0.1% peptone and spread on MRS agar. Afterwards, the plates were incubated at 37^0C for 48 h. The colonies from extensive growth plates were picked by following Harrison-disc method (Harrigan and McCance, 1976). Then the gram staining of isolated bacteria was done considering Tripathi and Sapra (2020) method. Initially, smear was prepared by spreading minimum colony and heat fixed on microscopic slide. First, crystal violet was dropped on the smear and allowed to for 10-60 seconds. After that it was poured off and excessive amount was rinsed with running water. Secondly, iodine solution was applied on the smear and allowed for 10-60 seconds. Then the solution was poured off and drained the excessive amount by running water. Thirdly, the decoloring agent (i.e., ethanol) was added and poured off after 5 seconds. Finally, basic safranin solution was added and allowed for 40-60 seconds. After that, it was drained with running water. Gram positive (+) strains were identified by observing the purple color under microscope. The gram positive (+) rod shaped colonies were re-streaked on MRS agar containing 0.3% $CaCO_3$ for screening most probable LAB considering Wu *et al*., (2012) method. The colonies that created clear zone in the MRS agar plate were selected and stored in MRS broth containing 50% glycerol at -20^0C for future analysis.

3.4 Determination of potential probiotics

3.4.1 Determination of hydrophobicity

Hydrophobicity of isolates was analyzed as described in Khullar *et al.*, 2022 method. Initially, fresh culture at 10^7-10^8 CFU / ml was centrifuged at 6000g for 5 min to separate cell pellet. Cell pellet was allowed to wash twice by phosphate buffer (PBS) and resuspended it in the same buffer. The absorbance of aqueous phase was measured at 600 nm. After that, an equal volume of organic solvent (xylene) was

added in the cell suspension and mixed vigorously for 2 mins. Cell suspension was incubated at laboratory temperature for 1 hour at steady state. After phase separation, the absorbance of aqueous phase was measured at 600 nm.

Cell surface hydrophobicity (%) = $(A_o - A_1) / A_0 \times 100$

A_o = Absorption before mixing with hydrocarbons

A_1 = Absorption after mixing with hydrocarbons

3.4.2 Determination of in-vitro gastric acid and bile salt tolerance

Probiotics must have the potential to survive at gastrointestinal condition. Thus, probiotic properties of isolated bacterial samples were analyzed under simulated gastrointestinal condition *in vitro* considering Gbassi *et al*., (2009) method with minor modifications.

Briefly, one or two colonies of isolates were put in MRS broth and allowed it to incubate at 37^{o}C for 24 hours prior to the analysis. 1 ml of fresh culture was mixed with 20 ml PBS buffer and incubated at 37^{o}C for 20 minutes in shaking incubator (100 rpm). After incubation, cell viability was counted by placing the samples on MRS plate by following spread plate method. In the same culture, 9 ml of simulated gastric fluid (SGF: mixing of NaCl 9 g/l and pepsin 3 g/l; adjusted pH at 1.8) was mixed properly and allowed to incubate at 37^{o}C for 2 hours at shaking condition. Cell viability was checked every hour. After 2 hours, 9 ml of simulated intestinal fluid (SIF: mixing of NaCl 9 g/l, pancreatin 10 g/l, bile 3 g/l, and lipase 0.03 g/l; adjusted pH near 6.8) was added in the same culture, mixed vigorously, and allowed to incubate at 37^{o}C for 3 hours at shaking condition. Cell viability was checked every hour.

3.4.3 Antimicrobial activity

The isolates that showed hydrophobicity and acid-bile tolerance were selected to analysis antimicrobial activity. Antimicrobial activity was performed by agar well diffusion method considering Khullar *et al*., (2022). At first, *Escherichia coli* ATCC25922 was spread on nutrient agar by cotton swap to create the lawn prior to making well. After drying of the plate, 100μl of fresh MRS culture of isolates was dropped in three different wells and let it dry in lab temperature. After drying, all the plates are incubated at 37^{o}C for 24 hours to see the clear zone. After incubation, the

antimicrobial activity of the colonies was measured by measuring the diameter of clear zone in each plate.

3.5 Determination of *in vitro* antioxidant activity of isolated bacteria

3.5.1 Preparation of sample

Fresh culture of isolates was used prior to every analysis. For getting fresh culture, isolated bacterial samples were cultured in MRS broth at 37^0C for 18 h before each analysis. After incubation, intact cells and fermented supernatant were separated by centrifugation at 6000g for 10 min at 4^0C. Isotonic saline (0.9%) was used for washing the sample three times and sample was resuspended in equal volume of isotonic saline. Finally, the concentration of cell pellet was adjusted to 10^8 CFU/ml.

3.5.2 DPPH free radical scavenging activity

Antioxidant activity was determined as mentioned in Tang *et al*., (2017) method. Briefly, 1 ml of fermented supernatant or intact cells was added to the 0.2 mM ethanolic DPPH of same volume. The solution was mixed thoroughly and kept in the dark environment for 30 min at ambient temperature. The control sample contained 1 ml of deionized water with the same amount of ethanolic DPPH solution (0.2 mM). Blank group was the 1 ml of ethanol rather than DPPH solution. After incubation, the mixed solution was centrifuged for 10 min and the supernatant was measured at the absorbance of 517 nm.

$$\text{Scavenging activity (\%)} = [1-(A_{\text{sample}} - A_{\text{blank}}) / A_{\text{control}}] \times 100$$

3.5.3 Reducing ability

Isolate that had all the potential probiotic properties was chosen for reducing ability. The reducing ability of isolated lactic acid bacteria was analyzed considering Lin and Yen (1999) method with minor alternations. Briefly, 0.5 ml of fermented supernatant or intact cells was mixed with 1% of 0.5 ml potassium ferricyanide and 0.5 mL of phosphate buffer saline (PBS) at pH 6.6. The mixed solution was gone through subsequent incubation at 50 °C for 20 min and quick cooling. After cooling, 0.5 ml of 10% trichloroacetic acid (TCA) will be mixed and centrifuged for 5 min at 3000g to separate pellet and supernatant. 1 ml of separated supernatant was mixed with same volume of 0.1% ferric chloride and measured the absorbance at 700 nm

after 10 min of reaction time. L- Cysteine was used as reference to evaluate the reducing ability.

3.5.4 Lipid peroxidation inhibition capacity

Potential probiotic isolate having potential probiotic properties was considered for lipid peroxidation inhibition capacity analysis. The lipid peroxidation inhibition capacity of lactic acid bacteria was determined based on the Kullisaar, Songisepp, and Mikelsaar (2003) method with little adjustments. Initially linoleic acid emulsion was prepared by the mixing of 1% of tween 20, 0.5% of linoleic acid and 98.5% of deionized water. Then the mixture for the reaction was made by adding 0.5 ml deionized water, 1 ml FeSO4 and 1 ml linoleic acid emulsion. This solution was mixed with 1 ml of fermented supernatant or intact cells and incubated in water bath at 37^0C for 2 hours. After incubation, 0.2 ml of trichloroacetic acid (4%) and 2 ml of thiobarbituric acid (0.8%) was added in the solution and allowed for incubation in water bath at 100^oC for 30 min and cooled in ice. After cooling, the solution was centrifuged at 4000g for 15 min to separate the supernatant and absorbance was measured at 532 nm. For the control sample, an equal volume of deionized water was used rather than the sample. The inhibition percentage will be expressed as:

$$\text{Inhibition capacity (\%)} = [(A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}}] \times 100$$

3.6 Identification of lactic acid bacteria

Identification of lactobacillus spp. was initialized by DNA extraction considering Dashti *et al.,* (2009) method with minor modifications. Briefly, two colonies of freshly incubated lactic acid bacteria was placed in test tube containing 200 µl of autoclaved distilled water and put it on freezer at -20^o C. After removing from freezer, it was boiled at 100 ^{o} C for 1 min in water bath. Then it was centrifuged at 1000 rpm for 5 mins and separated the supernatant for further analysis.

Identification of lactic acid bacteria was done as mentioned in Öz *et al*., (2017). Briefly, 30 µl of reaction mixture was prepared that was consisted of template DNA, Taq DNA polymerase, dNTPs, and PCR buffer. Identification of isolates was done by the selection of 16S rDNA coding region sequence and PCR amplification. Primers was used for amplification of 16S-rRNA gene: forward primer 27F (5'-AGA GTT TGA TCC TGGCTC AG-3') (Yi *et al*., 2019) and reverse primer 1492R (5'-

GGTTACCTTGTTACGACTT-3') (Tajabadi *et al.,* 2012). PCR products were analyzed by agarose gel electrophoresis and scanned in gel documentation system. PCR amplifications were sequenced, and sequenced result was evaluated by MEGA11 program and compared with GenBank database sequences.

3.7 Statistical analysis

All the experiments were performed three times and experimental data were analyzed by windows SPSS (version 28) software. All the data were expressed as mean ± Standard deviation. Duncan multiple range test ($p < 0.05$) was performed to determine significant differences among the samples.

Table 6 Fermented foods and their sources

Source	Fermented foods	Code	Salt concentration[1] (%)	Province
Plant	Phak kard dong A (Fermented green cabbage)	**NYC8**	8.0-16.0	Nakornsawan
	Phak kard dong B (Fermented green cabbage)	**NYNE7**	8.0-16.0	Udon
	Phak kard dong C (Fermented green cabbage)	**NYNE12**	8.0-16.0	Nakhon Ratchasima
	Phak kard dong D (Fermented green cabbage)	**NYNE14**	8.0-16.0	Nakhon Ratchasima
	Phak kard dong E (Fermented green cabbage)	**NYNE15**	8.0-16.0	Nakhon Ratchasima
	Bai miang A (Fermented tea leaf)	**NYN4**	0.1-1.5	Nan
	Bai miang B (Fermented tea leaf)	**NYN5**	0.1-1.5	Nan
	Bai miang C (Fermented tea leaf)	**NYN6**	0.1-1.5	Nan
	Nam hed A (Fermented Mushroom)	**NYNE1**	1.0 – 1.5	Nakhon Ratchasima
	Nam hed B (Fermented Mushroom)	**NYNE9**	1.0 – 1.5	Nakhon Ratchasima
	Nho mai dong A (Fermented Bamboo)	**NYW3**	0.5-6.4	Kanchanaburi
	Nho mai dong B (Fermented Bamboo)	**NYNE16**	0.5-6.4	Nakhon Ratchasima
	Nho mai dong C (Fermented Bamboo)	**NYNE17**	0.5-6.4	Nakhon Ratchasima
	Phak sian dong (Fermented wild spider flower)	**NYW4**	0.8-2.1	Kanchanaburi
	Ton hom dong (Fermented onion leaf)	**NYNE22**		Sakonnakorn

	Plasom A (Sour Fish) Banana leaf wrapper	**NYNE2**	4.0-10.7	Udon
	Plasom B (Sour Fish) Plastic wrapper	**NYNE3**	4.0-10.7	Udon
	Plasom C (Sour Fish) Plastic wrapper	**NYNE8**	4.0-10.7	Nakhon Ratchasima
	Plasom D (Sour Fish) Plastic wrapper	**NYNE10**	4.0-10.7	Nakhon Ratchasima
	Plasom E (Sour Fish) Plastic wrapper	**NYNE21**	4.0-10.7	Sakonnakorn
	Kung jom A (Fermented shrimp)	**NYNS1**	3.2 – 9.4	Udon
	Kung jom B (Fermented shrimp)	**NYNS2**	3.2 – 9.4	Udon
Animal	Kung jom C (Fermented shrimp)	**NYNE23**	3.2 – 9.4	Sakonnakorn
	Mum A (Beef sausage)	**NYNE4**	2.3 – 7.1	Udon
	Mum B (Beef sausage)	**NYNE19**	2.3 – 7.1	Sakonnakorn
	Pla kem (Salted fish)	**NYW1**	11.5 – 23.9	Kanchanaburi
	Pla ra (Salted fish)	**NYW2**	11.5 – 23.9	Kanchanaburi
	kao-mak-pra-ta-pean (Fermented rice in carp)	**NYNE11**		Nakhon Ratchasima
	Naem (Fermented Pork)	**NYNE18**	2.5-2.8	Nakhon Ratchasima
	Som sai pla (Fermented fish viscera)	**NYNE24**	13.5-25.3	Sakonnakorn

[1] (Yongsmith and Malaphan, 2016)

Result and Discussion

4.1 Isolation of lactic acid bacteria from Thai local fermented foods

Fermented foods were collected from different parts (central, north, north-east, and west) of Thailand. These collected foods (**Figure 4.1**) from different provinces can be categorized into two groups: plant and animal-based food. These fermented foods can be further divided into lactic acid fermented foods and complex fermented foods (protein and cellulose hydrolysate fermentation); based on fermentation process. Lactic acid fermentation naturally performed in Phak kard dong (fermented green cabbage), Phak sian dong (fermented wild spider flower), Nho mai dong (fermented bamboo), Plasom (sour fish); in contrast complex fermentation performed in Bai miang (fermented tea leaf), Kung jom (fermented shrimp), Mum (fermented beef sausage), Naem (fermented pork), Pla kem (salted fish), Som sai pla (Fermented fish viscera) and Pla ra (salted fish). Complex fermented food (Bai miang) also contain a little amount LAB that aided aroma generation (Wu *et al*., 2020) which made this food group a source of LAB.

Figure 4. 1 Fermented food sample

Kung jom

Nam hed

Mum

Plasom

Plasom

Pla kem

Som sai pla

Naem

Pla ra

kao-mak-pra-ta-pean

Figure 4.1: Fermented food samples (continue)

In this study, preliminary screening was done by MRS agar which contains polysorbate (sorbic acid) compound that limited the growth of other bacteria except lactic acid bacteria (Corry, Curtis, and Baird, 2003). This screening method was designed to isolate facultative anaerobic bacteria to get simple cultivation strains.

Consequently, screening was done by the inspection of cell morphology considering gram staining method. Only gram-positive (**Figure 4.2 and Appendix A 2**) rod shaped isolates were selected for the next analysis. Maximum number of gram-positive isolates were found in animal based fermented foods that includes Plasom B, Pla kem , Mum A, Plasom C, Plasom D, Som sai pla, and followed by Plasom A, Pla ra, Kung jom A, Kung jom B, and Kung jom C. Whereas in the plant- based fermented foods, maximum number of gram-positive isolates were found in Phak kard dong B and followed by Nho mai dong A, Bai miang B, Bai miang C, Phak kard dong A, Phak sian dong, Nam hed A, Phak kard dong D, Phak kard dong C, Phak kard dong E, Bai miang A, Nho mai dong B, and Nam hed B as shown in **Table 7**. Most of the isolates from all fermented foods contained more than 50% of gram-positive rod shaped except Kung jom A, Nho mai dong B, Nham hed B, Kao mark pla ta pean, Naem which was further streaked on MRS agar plate containing 0.3% $CaCO_3$ to screen the potential LAB. Colony generating lactic acid was tested by observing clear zone from the reaction of the acid and $CaCO_3$ (Wu *et al.*, 2012). The clear zone was observed as shown in **Figure 4.3 and Appendix A 3** in eighteen isolate colonies (NYC8, NYNS 1 B, NYNS 1 S, NYW2, NYNS2B, NYNS2S, NYNE2B, NYW1Y, NYW4, NYNE8.1, NYNE8.2, NYNE8.3, NYNE9.1, NYNE10.2, NYNE10.3, NYNE10.6, NYNE23.1, and NYNE23.5) that could be specified as LAB isolates. Normally, animal-based foods (especially dairy products) are the major sources for LAB screening, but plant sources have recently become an interesting option because of the availability of LAB in some specific plant-based foods.

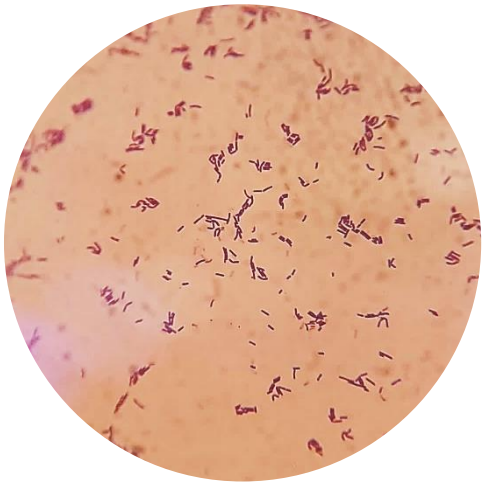

Figure 4. 2 Gram staining of isolated sample

Mostly LAB in fermented foods including Phak kard dong, Phak sian dong, and Plasom etc imparts sour taste in the food. LAB growth and survival depends on the lactic acid fermentation in fermented foods which is affected by several factors (Hofvendahl and Hahn–Hägerdal, 2000). In the complex fermentations, some microbes, like *Bacillus spp,* yeast, and mold, could synthesize some enzymes such as cellulase which initially enhance the LAB growth and lower the pH of the environment by producing lactic acid. Over time the functionality of the cellulase enzyme decreases under low pH which minimize the availability of water-soluble carbohydrates for LAB growth. At lower pH, the unavailability of water-soluble carbohydrates leads LAB to synthesize lactic acid to acetic acid which increase the pH of the environment and minimize the growth of LAB in the fermented ecosystem (Parvin, Wang, and Nishino, 2010). Moreover, in this study, LAB could be found more in Plasom B and Plasom E (wrapped with sealed plastic film) compared to Plasom A (wrapped with banana leave), Plasom C, Plasom D (unsealed plastic wraper) as shown in (**Table 7**). This demonstrated that packaging could be a key factor associated with LAB growth, particularly, effect from their oxygen barrier properties. The anaerobic condition is expected to initiate the growth of LAB compared to aerobic condition. However, the rapid growth of LAB at anaerobic conditions could cause faster pH drops and this low pH environment could limit the growth of LAB in that environment (Adamberg *et al*., 2003) as observed in Plasom B and Plasom E wrapped in sealed plastic film. Besides these factors, fermentation time could also have noticeable effect on the viable count of LAB in fermented foods. Extended fermentation time lowers the pH which could narrow the probability of LAB presence in LAB containing food (Li *et al*., 2022).

In this study, daily consumable fermented foods were collected randomly from the local market. Some fermented foods (Bai miang, Plasom) in this study contained gram positive isolates but no potential LAB was observed which can be attributed to several factors such as fermentation time and temperature along with fermentation environment. Besides these factors, composition of raw materials and the packaging type also affected the LAB population. This observation demonstrated that fermented food as a good source of potential LAB, key factors such as fermentation time,

temperature, manufacturing process, raw materials, and storage condition should be criteria for selection.

Figure 4. 3 MRS agar plate containing 0.3% $CaCO_3$

Table 7 Food sources and Profile of isolate characterization

Source	Fermented foods	Code	Salt concentration (%)	Province	Gram staining (No. of isolate)		$CaCO_3$ positive (No. of isolate)	DPPH inhibition (No. of isolate)		Probiotic property (No. of isolate)		
					Gram positive	Gram negative		Supernatant	Pellet	Hydrop hobicity	Acid-bile tolerance	Antimicrobial activity
Plant	Phak kard dong A (Fermented green cabbage)	**NYC8**	8.0-16.0	Nakornsawan	4	1	1	1	1	1	1	1
	Phak kard dong B (Fermented green cabbage)	**NYNE 7**	8.0-16.0	Udon	6	2	0	N/A	N/A	N/A	N/A	N/A
	Phak kard dong C (Fermented green cabbage)	**NYNE 12**	8.0-16.0	Nakhon Ratchasima	3	2	0	N/A	N/A	N/A	N/A	N/A
	Phak kard dong D (Fermented green cabbage)	**NYNE 14**	8-16	Nakhon Ratchasima	4	2	0	N/A	N/A	N/A	N/A	N/A
	Phak kard dong E (Fermented green cabbage)	**NYNE 15**	8-16	Nakhon Ratchasima	3	2	0	N/A	N/A	N/A	N/A	N/A
	Bai miang B (Fermented tea leaf)	**NYN5**	0.0 -5.0	Nan	5	0	0	N/A	N/A	N/A	N/A	N/A
	Bai miang A (Fermented tea leaf)	**NYN4**	0.0 -5.0	Nan	2	2	0	N/A	N/A	N/A	N/A	N/A

	Fermented foods	Code	Salt concentration (%)	Province	Gram staining (No. of isolate)		$CaCO_3$ positive (No. of isolate)	DPPH inhibition (No. of isolate)		Probiotic property (No. of isolate)		
					Gram positive	Gram negative		Supernatant	Pellet	Hydrop hobicity	Acid-bile tolerance	Antimicrobial activity
Plant	Bai miang B (Fermented tea leaf)	**NYN5**	0.0 -5.0	Nan	5	0	0	N/A	N/A	N/A	N/A	N/A
	Bai miang C (Fermented tea leaf)	**NYN6**	0.0 -5.0	Nan	5	1	0	N/A	N/A	N/A	N/A	N/A
	Nam hed A (Fermented Mushroom)	**NYNE 1**	1.0 – 1.5	Nakhon Ratchasima	3	1	0	N/A	N/A	N/A	N/A	N/A
	Nam hed B (Fermented Mushroom)	**NYNE 9**	1.0 – 1.5	Nakhon Ratchasima	1	2	1	1	1	0	N/A	N/A
	Nho mai dong A (Fermented Bamboo)	**NYW3**	8.0-16.0	Kanchanaburi	5	2	0	N/A	N/A	N/A	N/A	N/A
	Nho mai dong B (Fermented Bamboo)	**NYNE 16**	8.0-16.0	Nakhon Ratchasima	1	3	0	N/A	N/A	N/A	N/A	N/A
	Nho mai dong C (Fermented Bamboo)	**NYNE 17**	8.0-16.0	Nakhon Ratchasima	2	2	0	N/A	N/A	N/A	N/A	N/A
	Phak sian dong (Fermented wild spider flower)	**NYW4**	8.0-16.0	Kanchanaburi	3	2	1	1	1	1	0	N/A
	Ton hom dong (Fermented onion leaf)	**NYNE 22**	N/A	Sakonnakorn	1	1	0	N/A	N/A	N/A	N/A	N/A

Source	Fermented foods	Code	Salt concentration (%)	Province	Gram staining (No. of isolate)		$CaCO_3$ positive (No. of isolate)	DPPH inhibition (No. of isolate)		Probiotic property (No. of isolate)		
					Gram positive	Gram negative		Supernatant	Pellet	Hydrophobicity	Acid-bile tolerance	Antimicrobial activity
Animal	Plasom A (Sour Fish) Banana leaf wrapper	**NYNE 2**	2.0-2.5	Udon	4	2	1	1	1	1	0	N/A
	Plasom B (Sour Fish) Sealed Plastic wrapper	**NYNE 3**	2.0-2.5	Udon	8	5	0	N/A	N/A	N/A	N/A	N/A
	Plasom C (Sour Fish) Unsealed Plastic wrapper	**NYNE 8**	2.0-2.5	Nakhon Ratchasima	7	3	3	3	3	0	1	N/A
	Plasom D (Sour Fish) Unsealed Plastic wrapper	**NYNE 10**	2.0-2.5	Nakhon Ratchasima	7	2	3	3	3	-	-	N/A
	Plasom E (Sour Fish) Sealed Plastic wrapper	**NYNE 21**	2.0-2.5	Sakonnakorn	2	2	0	N/A	N/A	N/A	N/A	N/A
	Kung jom A (Fermented shrimp)	**NYNS 1**	5.0-10.0	Udon	2	3	2	2	2	0	0	N/A
	Kung jom B (Fermented shrimp)	**NYNS 2**	5.0-10.0	Udon	2	2	2	2	2	1	0	N/A

Source	Fermented foods	Code	Salt concentration (%)	Province	Gram staining (No. of isolate)		$CaCO_3$ positive (No. of isolate)	DPPH inhibition (No. of isolate)		Probiotic property (No. of isolate)		
					Gram positive	Gram negative		Supernatant	Pellet	Hydrophobicity	Acid-bile tolerance	Antimicrobial activity
Animal	Kung jom C (Fermented shrimp)	**NYNE 23**	5.0-10.0	Sakonnakorn	2	3	2	2	2	0	0	N/A
	Mum A (Beef sausage)	**NYNE 4**	2.0-2.5	Udon	8	2	0	N/A	N/A	N/A	N/A	N/A
	Mum B (Beef sausage)	**NYNE 19**	2.0-2.5	Sakonnakorn	2	4	0	N/A	N/A	N/A	N/A	N/A
	Pla kem (Salted fish)	**NYW1**	12.0-25.0	Kanchanaburi	8	2	1	1	1	1	0	N/A
	Pla ra (Salted fish)	**NYW2**	12.0-25.0	Kanchanaburi	4	2	1	1	1	1	0	N/A
	kao-mak-pra-ta-pean (Fermented rice in carp)	**NYNE 11**		Nakhon Ratchasima	1	3	0	N/A	N/A	N/A	N/A	N/A
	Naem (Fermented Pork)	**NYNE 18**	2.5-2.8	Nakhon Ratchasima	2	4	0	N/A	N/A	N/A	N/A	N/A
	Som sai pla (Fermented fish viscera)	**NYNE 24**	13.5-25.3	Sakonnakorn	4	3	0	N/A	N/A	N/A	N/A	N/A

4.2 Potential probiotic properties

Probiotics must pass through the digestion tract prior providing health benefits to their host. The acidic condition of stomach provides a natural barrier to screen out pathogenic microorganisms but not probiotics. After reaching the intestine, probiotic starts accumulating to the epithelial cell of intestine and stimulates immunity system and most importantly inhibits the growth of harmful bacteria (Nikoskelainen *et al*., 2003). Probiotics maintain sound health of gastro-intestinal (GI) tract by stimulating gut microbiota with synthesis and releasing antimicrobial compounds. These functioning manners of probiotics are interlinked with the health promoting effects (Parvez *et al*., 2006). Thus, analysis of probiotic properties of the isolates were initialized to establish a potential candidate of probiotics.

Hydrophobicity is considered as one of the major probiotic properties which reflects the adhesion of probiotic candidates with the epithelial cell wall of intestinal mucosa. This adhesion is necessary to exhibit health promoting effects and stimulation of immune systems. Potential probiotic strain must have hydrophobicity of 40% to maintain adhesion and interaction with the epithelial cell wall of intestinal mucosa (Del Re *et al.,* 2000). Conventionally, cell surface hydrophobicity can be analyzed using xylene as an organic solvent as it is independent of electrostatic interaction (Martins *et al*., 2009). From this study, maximum hydrophobicity was observed in NYC8 followed by NYNS2B, NYW2, NYW4, NYNE2B, NYW1Y and others respectively as shown in **Table 8**. The hydrophobicity of NYC8 and NYNS2B strains were 92.56% and 48.83% which exceeded the least requirement of potential probiotic (40%). Results of NYC8 and NYNS2B reflected better adhesion with the mucosa cell wall and survivability in the gastrointestinal tract (Kos *et al.,* 2003). Hydrophobicity of LAB comes from their surface properties that is contributed by (lipo-)teichoic acids, polysaccharide interaction with protein and. S-layer protein (Deepika *et al*., 2009).

Table 8 Potential probiotic properties of targeted isolates

Fermented Foods	Isolates	Hydrophobicity (%)	Acid tolerance (CFU/ml)	Bile tolerance (CFU/ml)	Antimicrobial activity (Diameter, m)
Phak kard dong A (Fermented green cabbage)	**NYC 8**	92.56	2.49×10^{5}	1.84×10^{4}	2.12×10^{-2}
Phak sian dong (Wild spider flower)	**NYW4**	24.04	-	-	N/A
Nam hed B (Fermented Mushroom)	**NYNE9.1**	-2.44	-	-	N/A
Kung jom A (Fermented shrimp)	**NYNS1S**	-79.08	-	-	N/A
Kung jom A (Fermented shrimp)	**NYNS1B**	-43.34	-	-	N/A
Kung jom B (Fermented shrimp)	**NYNS2B**	48.83	-	-	N/A
Kung jom B (Fermented shrimp)	**NYNS2S**	-52.33	-	-	N/A
Kung jom C (Fermented shrimp)	**NYNE23.1**	-7.89	-	-	N/A
	NYNE23.5	-25.68	-	-	N/A

Table 8: Continue

Fermented Foods	Isolates	Hydrophobicity (%)	Acid tolerance (CFU/ml)	Bile tolerance (CFU/ml)	Antimicrobial activity (Diameter, m)
Plasom A (Sour Fish) Banana leaf wrapper	**NYNE2B**	4.50	-	-	N/A
Plasom C (Sour Fish) Plastic wrapper	**NYNE8.1**	-11.20	-	-	N/A
	NYNE8.2	8.38	-	-	N/A
	NYNE8.3	-34.29	-	-	N/A
Plasom D (Sour Fish) Plastic wrapper	**NYNE10.2**	8.59	-	-	N/A
	NYNE10.3	-18.19	-	-	N/A
	NYNE10.6	18.69	-	-	N/A
Pla kem (Salted fish)	**NYW1Y**	1.61	-	-	N/A
Pla ra (Salted fish)	**NYW2**	31.85	-	-	N/A

After hydrophobicity analysis, isolates having 40% hydrophobicity were mixed with PBS before introducing acid-bile solution to reduce the cell deformation, homogenous mixing and maintaining the pH. It was found that only NYC8 isolate out of eighteen isolates showed the resistance in both simulated gastric and intestine environment. Initially the concentration of NYC8 was 2.45×10^6 CFU/ml (**Table 8**) which was reduced to 2.49×10^5 after two hours of gastric environment and after three hours of bile treatment, it came down to 1.84×10^4 CFU/ml. Bile tolerance of NYC8 isolate could be attributed to bile tolerant protein synthesis adaptation over the time against oxidative damage (Ruiz *et al*., 2013; Russo *et al*., 2012). The overall viability of NYC8 isolate was observed more than 50 % which made it potential candidates of probiotics.

Antimicrobial activity of probiotic is another essential probiotic property which reflects the potentiality of a probiotic bacteria to inhibit the growth of deleterious pathogenic bacteria in the intestine. In this study, antimicrobial property was initially screened by testing on *E. coli ATCC25922.* Only NYC8 showed inhibitory action on *E. coli* by creating clear zone of 2.12×10^{-2} m on MRS agar plate. Since the inhibition capacity of LAB is associated with the generation of antimicrobial substances that includes bacteriocins, organic acid, and hydrogen peroxide (Fei *et al.*, 2018; Servin, 2004), the inhibitory activity of this LAB isolates is required further investigation under other pathogens like *Salmonella Typhimurium* ATCC1331, *Bacillus subtilis* subsp. *spizizenii* (ATCC® 6633™), *Staphylococcus aureus* ATCC 25923 etc. (Khullar *et al.,* 2022). Further study like hemolytic activity, antimicrobial susceptibility, *in vivo* animal trials of this isolate is required for the confirmation of probiotic properties.

4.3 *In vitro* antioxidant activity of isolated bacteria

4.3.1 DPPH scavenging capacity

DPPH scavenging capacity of the $CaCO_3$ positive isolates were further analyzed to screen the potential LAB having antioxidant activity. DPPH scavenging capacity was analyzed for supernatant and pellet separately as they have different mechanisms for antioxidant activity. After analysis of DPPH scavenging capacity, all isolates were

found to show more than 70% antioxidant property (**Figure 4.4**) except the isolate NYNE10.3 supernatant (49.12%) and NYNE23.1 pellet (58.08%). Antioxidant activity of fermented supernatant was found higher about 99% in NYNS1S isolate followed by NYNS1B, NYC8, NYW1Y, NYNE9.1, NYNE10.6, NYNE10.2, NYNE8.1, NYNS2B, NYNE2B, NYNS2S, NYW2, NYNE23.1, NYNE8.3, NYNE8.2, NYNE23.5, NYW4, and NYNE10.3 isolates. The antioxidant activity of fermented supernatant comes from the extracellular polysaccharides e.g., pyruvate which non-enzymatically scavenge some sort of free radicles (van Niel, Hofvendahl, & Hahn-Hägerdal, 2002). On the other hand, NYNS2S isolate showed highest antioxidant activity in pellet followed by NYNS2B, NYNE8.3, NYW4, NYNE8.1, NYNS1S, NYNE8.2, NYNE8.2, NYNE10.2, NYC8, NYW2, NYNE10.3, NYNS1B, NYW1Y, NYNE9.1, NYNE2B, NYNE10.6, NYNE23.5, and NYNE23.1. In the pellet, antioxidant activity is generated by many non-enzymatic factors. Non enzymatic antioxidant activity initiates by the presence of Mn^{2+}, -SH group of amino acid side chain (Horsburgh *et al*., 2002) and the surface-active compounds that includes polysaccharides, protein, and lipoteichoic acid (Li *et al*., 2012; Yi *et al*., 2009). Overall antioxidant activity was found higher in animal based fermented foods compared to the plant based fermented foods. In general, animal-based foods are composed of high amount of lipids which undergoes peroxidation and generates cytotoxic compounds that include hydroperoxides, hydroxy alkanals malondialdehyde etc. (Kanner and Lapidot, 2001). The screened isolates showed an antioxidant defense system to survive the cytotoxic environment which elevated its antioxidant property. Tang *et al*., 2017 reported the DPPH scavenging capacity of isolated *Lactobacillus plantarum* MA2 from kefir was found 85.57 ± 0.44 for supernatant and 83.93 ± 1.54 for pellet under same protocol as present study. Li *et al*., 2018 also reported the DPPH scavenging activity of isolated *Lactobacillus plantarum* KLDS1.0601 from dairy food was found 47.67 ± 1.36 for pellet. Commercial anti-aging probiotic supplements contain a blend of probiotic strains where *Lactobacillus plantarum* 90 and *Lactobacillus acidophilus* 85 are the dominant strains. DPPH scavenging capacity of *Lactobacillus plantarum* 90 and *Lactobacillus acidophilus* 85 from different fruit juices were ranged between 65-90% based on the food source and fermentation time (Wang *et al.,* 2022; Wu *et al.,* 2020). In this study, DPPH scavenging activity of

isolates from Thai fermented food showed relatively higher antioxidant property compared to those in previous studies. This could be due to several factors including manufacturing processes such as salt content, antimicrobial activity from raw materials and fermented temperature. These factors could cause stress conditions which affected the bacterial strains to adapt and express the defense mechanism, for example increasing the cell wall thickness and exopolysaccharide to protect the bacterial cell from stress environments. Particularly, there are some reports which demonstrated that temperature could be a key factor associated with this defense mechanism. The higher temperature could cause stress and minimize the growth of LAB but stimulate the generation of exopolysaccharide to neutralize the stress.

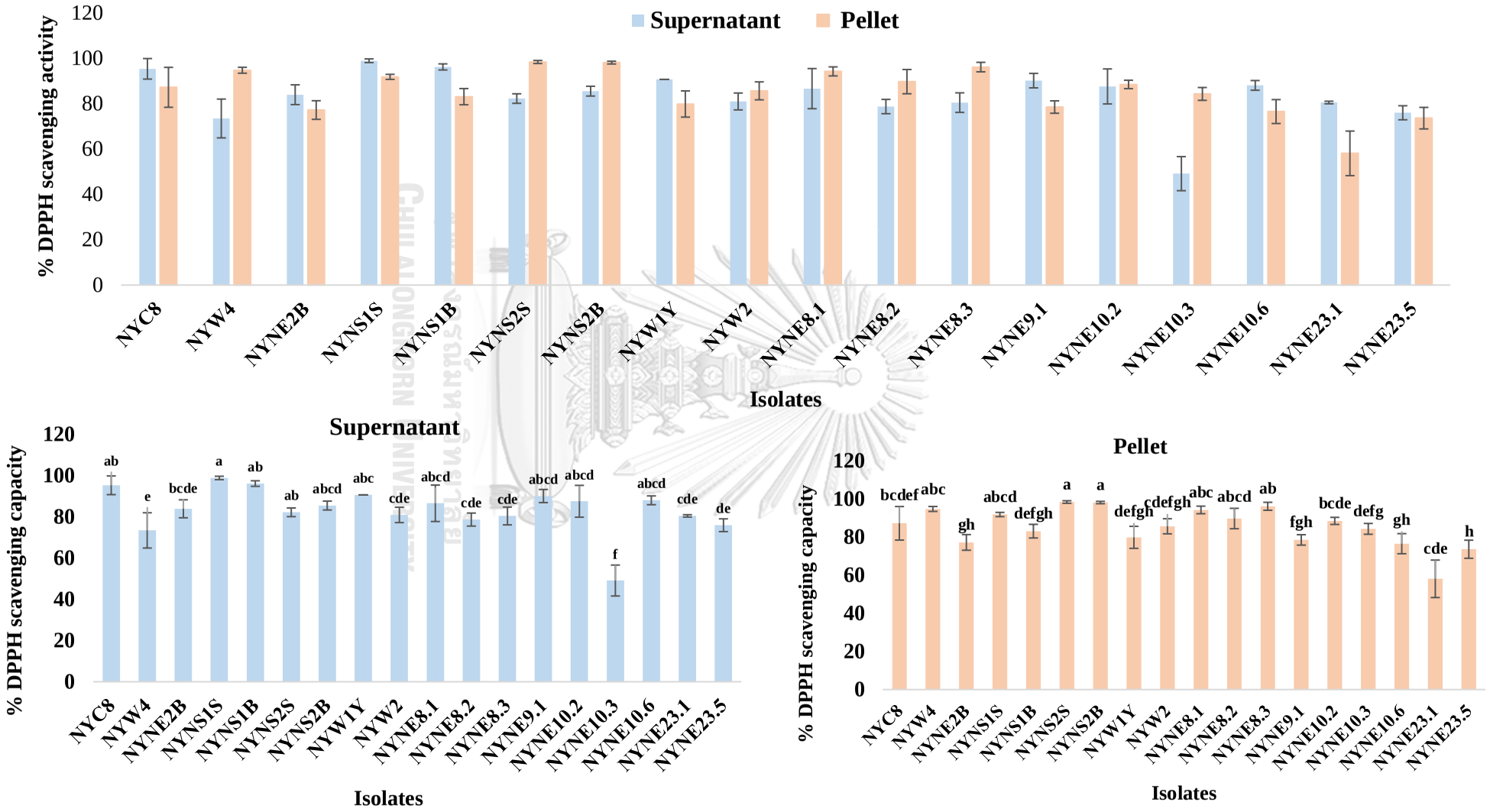

Figure 4. 4 DPPH scavenging capacity of isolated samples (Supernatant and pellet)

4.3.2 Reducing ability

Reducing ability is another indication of antioxidant capacity in microbial cells which is identified by the reduction of potassium ferricyanide. Antioxidants in cells convert the Fe^{3+} of potassium ferricyanide to Fe^{2+} which can be identified by the color changes at the end of reaction. Reducing ability in LAB is generated possibly from the availability of antioxidants and proteins in the intracellular space (Lin and Yen, 1999). NYC8 isolate having all the potential probiotic properties was considered for reducing ability analysis. Reducing ability of isolated NYC8 strain was observed equivalent of 778.62 µM L-cysteine hydrochloride for supernatant and 195.91 µM L-cysteine hydrochloride for pellet (**Table 9**). Tang *et al.,* (2017) reported the reducing ability of *Lactobacillus plantarum* MA2 cell free extract was 154.1 µM L-cysteine hydrochloride considering same protocol. Reducing ability of *Lactobacillus acidophilus* KLDS 1.0732 intact cell was observed 120.09 µM L-cysteine hydrochloride equivalent considering the same protocol (Li *et al.,* 2018). For the supernatant, no reducing ability was observed in both literatures mentioned above. But in the present study, there was an unusual high reducing ability which might be the presence of reducing sugar (dextrose) in the MRS broth composition. Reducing ability of the present study was found higher compared to the previous studies. That was probably the effect of high antioxidant content of the isolated sample that converted Fe^{3+} of potassium ferricyanide to Fe^{2+} which elevated the reducing ability (Lin and Yen, 1999).

4.3.3 Lipid peroxidation inhibition capacity

Lipid peroxidation is one of the major oxidation reactions in a system which generates toxic compounds. Hydroperoxides are the initial compounds that generate because of lipid autoxidation. Hydroperoxides are considered toxic because they have potential to damage DNA structure. Beside these compounds, malondialdehyde (MDA) is a secondary product of lipid peroxidation which is a biomarker of oxidative stress. It is directly associated with the antioxidant defense system (SOD, GPx, T-AOC) which reflects the presence of free radicals in a system (Li *et al.,* 2018). MDA presence in a system causes degradation of biological molecules (DNA, protein, and others) because of its highly reactive nature. Additionally, MDA causes alteration of

cell morphology, creation of cell vacuolization, and minimization of protein synthesis (Lin and Yen, 1999). Based on these considerations, inhibition of unsaturated linoleic acid peroxidation of NYC8 isolate was analyzed. NYC8 isolate was chosen for lipid peroxidation inhibition capacity as it showed all potential probiotic properties. Present study showed the inhibition of lipid peroxidation for NYC8 isolate 4.34% for supernatant and 44.34% for pellet (**Table 9**) which is quite similar as found by Lin *et al.,* (2018) with *Lactobacillus plantarum* AR113 pellet of 46.94% and with *Lactobacillus bulgaricus* CCFM29 pellet of 44.4% by Zhai et al., (2015).

Table 9 Reducing ability and lipid peroxidation inhibition capacity of NYC8 isolate

	Supernatant	**Pellet**
Reducing ability (μM L-cysteine equivalent)	778.62 ± 6.12	195.91 ± 2.26
Lipid peroxidation inhibition capacity (%)	4.34 ± 0.26	44.34 ± 0.54

4.4 Identification of potential probiotics

In the present study, NYC8 showed clear zone on MRS agar plate, antioxidant activity and all the primary potential probiotic properties. Therefore, this targeted isolate was further identified through sequencing analysis and compared with GenBank database. For sequencing, 1500 bp amplified fragment was used. After sequencing and comparing with GenBank database, 100% homology was observed between NYC8 isolate with other two strains *Lactiplantibacillus plantarum* ATCC 14917^T (ACGZ01000098) and *Lactiplantibacillus argentoratensis* DSM 6365^T (CP032751). Even though the phenotypic properties of *Lactiplantibacillus argentoratensis* DSM 6365^T (CP032751) (formerly *Lactobacillus argentoratensis*) and *Lactiplantibacillus plantarum* ATCC 14917^T (ACGZ01000098) (formerly *Lactobacillus plantarum*) are almost similar which is difficult to separate by 16S rDNA sequencing. But their functionalities are different in terms of fermentation of organic compounds. *Lactiplantibacillus argentoratensis* DSM 6365^T (CP032751) has the capability to ferment a wide range of carbohydrates including cellobiose as well as fructose which eventually turns into mannitol (Syrokou *et al.,* 2022). Strains having similarity after 16S rDNA sequencing can be screened initially by IR Biotyper (IRBT)

which works considering Fourier transform infrared (FTIR) system. For confirmation of a strain, the dendograms of IRBT are compared with whole-genome sequencing (WGS), pulsed-field gel electrophoresis (PFGE), and multilocus sequence typing (MLST) data to separate closely related strains under same genus (Li *et al.*, 2022). Moreover, whole genome sequence will provide all genes associated with the targeted species and association genes with the health promoting activities (Dash and Das,

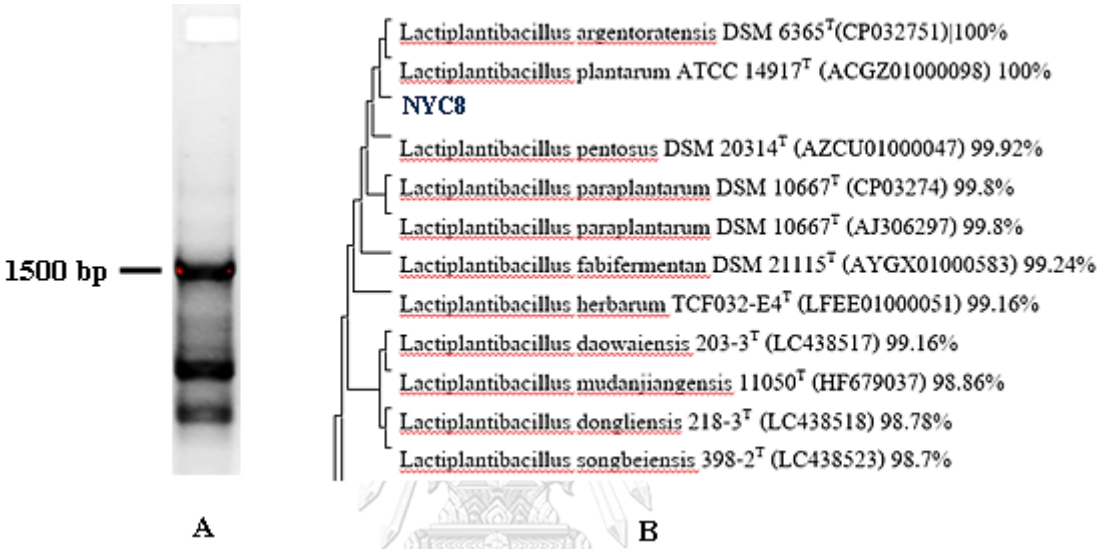

Figure 4. 5 **A)** Agarose gel electrophoresis and **B)** Phylogenetic tree of isolate NYC8 2022).

After analyzing all the fermented foods from Thai local makkets, only one isolate NYC8 was showing potential probiotic properties. Generally, fermented foods from Thai local makrets are not prepared according to a standard protocol which narrow down the probability of getting a potential probiotic candidate. Besides this, isolation technique was another factors for the less number. Analyzing quantity of a sample was too small to represent the large quantity which limited the isolation of potential candidates. However, NYC8 has potential to be a probiotic strains with antioxidant properties and could be develop as a commercial starins. The functionality of NYC8 in terms of antioxidant activity and potential probiotic prooperties were found higher compared to the commercial strains available in the market.

Conclusion

According to the information obtained in this study, Thai traditional fermented foods could be the source of LAB with significant antioxidant activity which might be developed as potential probiotic stains. This observation also demonstrated fermented food as a source of potential probiotic, key factors such as fermentation stage, manufacturing process, composition of raw material, and storage condition should be criteria for source of isolate selection. The isolate, NYC8 could be a potential probiotic candidate which had noticeable antioxidant activity and preliminary probiotic properties. Therefore, this isolate could be further studied *in vivo* to observe its health promoting effects as a function of different non enzymatic (amino acids containing Mn^{2+} and -SH group) and enzymatic (SOD, CAT, and GPx) defense systems in the living organism at the presence of different genes and their expression level. These evaluations of the isolate, NYC8 might ease the application of it in industrial level for the preparation of functional foods and natural supplements.

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Appendix

Appendix-A1: Harrison-Disc method

Most common practice for selecting colony from a large population of microorganisms. Initially, microorganisms under area of number 1 was taken and the process continued to number 2, 3, and 4 marked areas until the desired number of microorganisms were selected. Colonies that were lying over the marked lines were ignored for analysis.

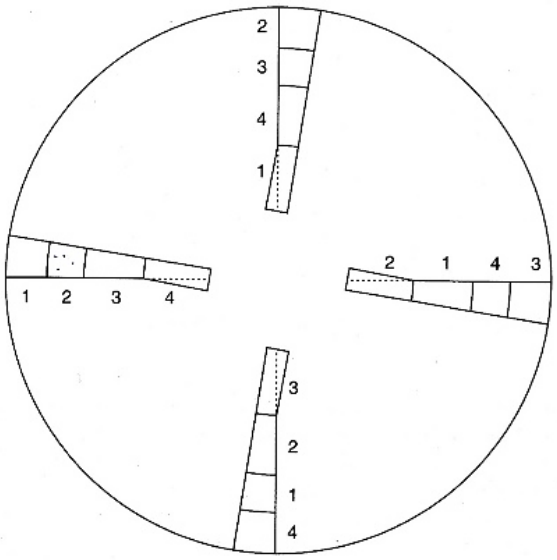


Figure A 1 Harrison disc

Appendix-A2: Gram staining of isolated samples

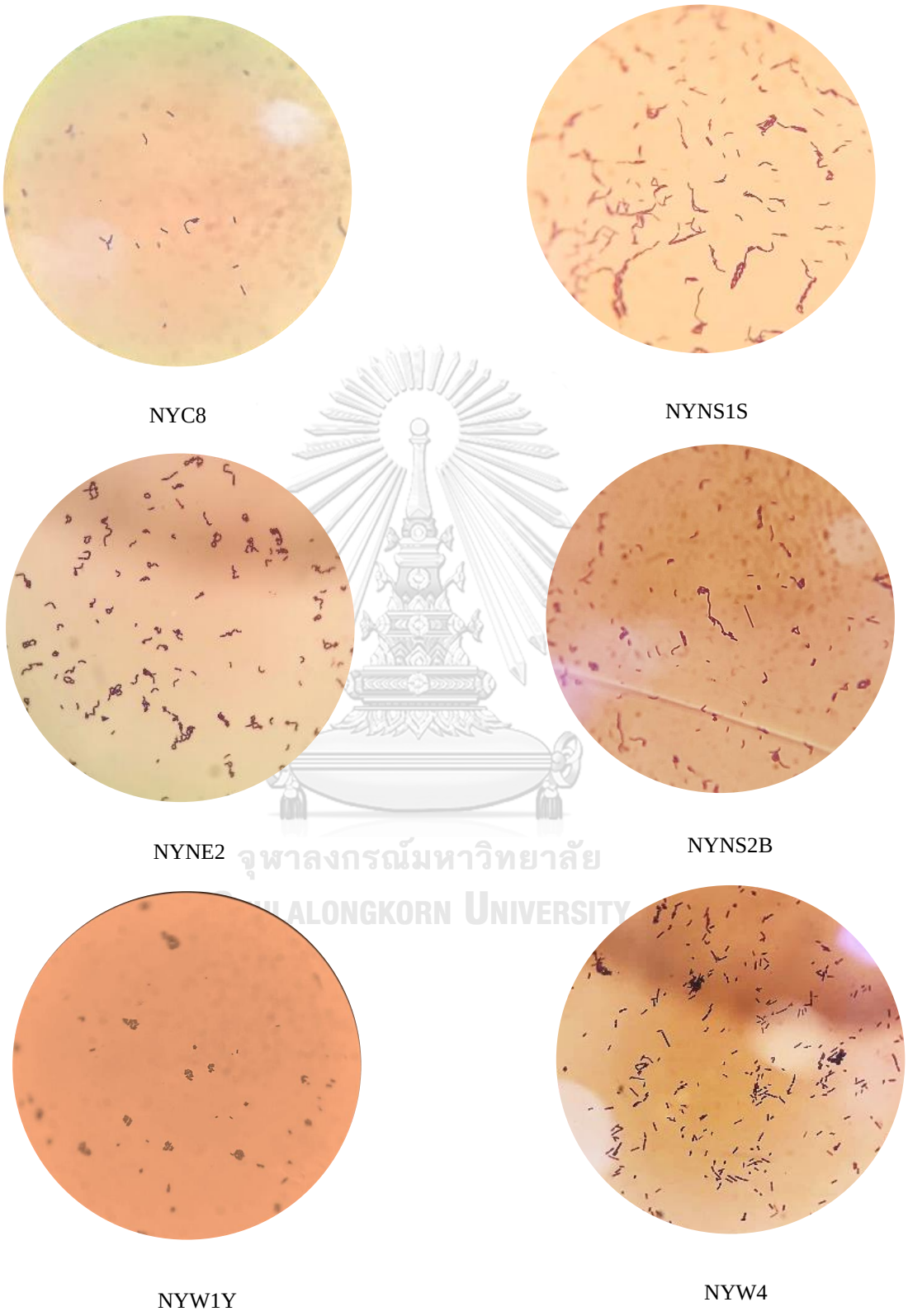
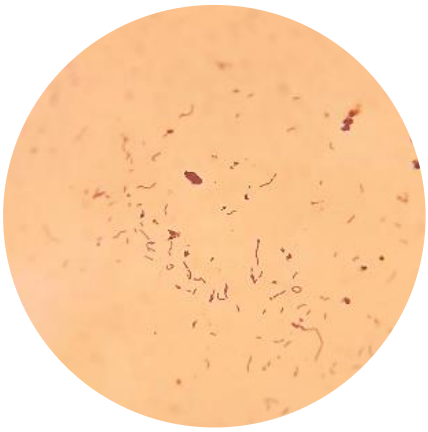
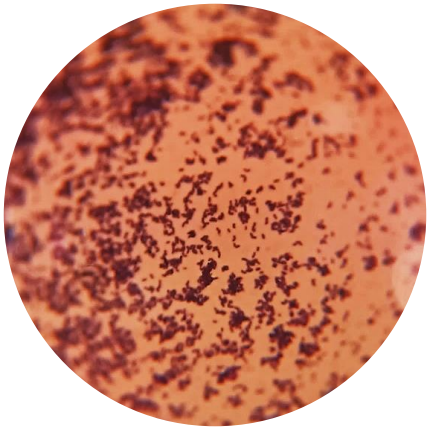


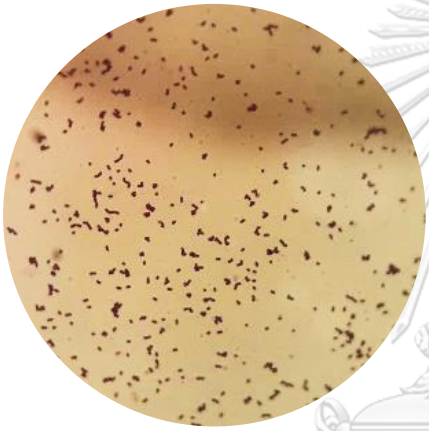
Figure A 2 Gram staining of isolated samples



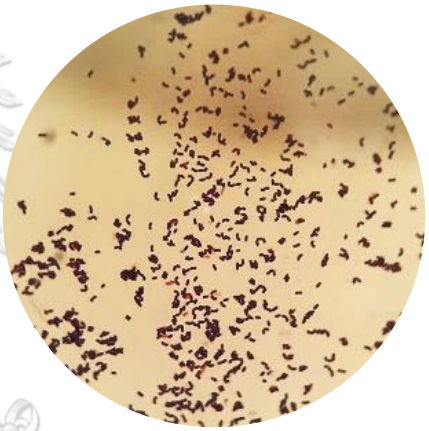
NYNS1B



NYNE8.1



NYNE8.2



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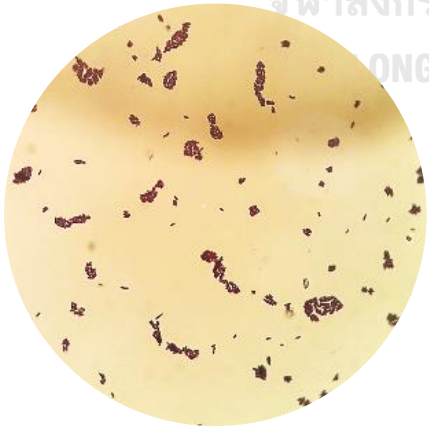
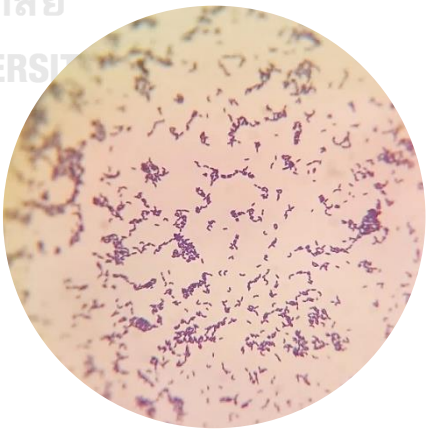
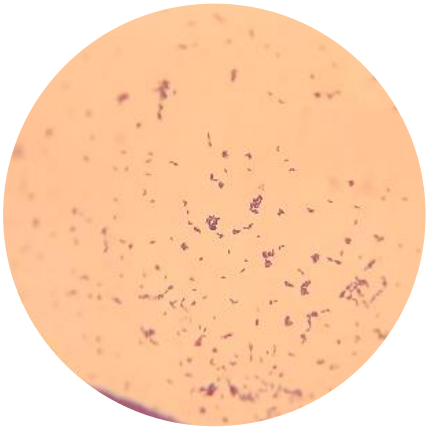


NYNE9.1

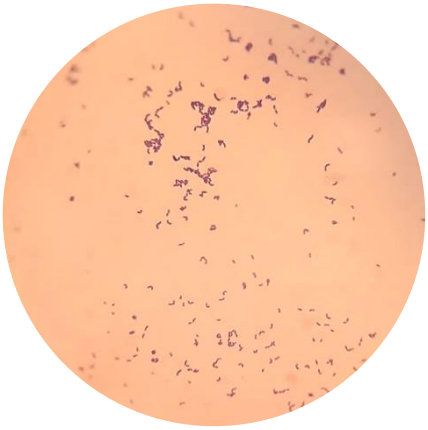


NYNE10.2

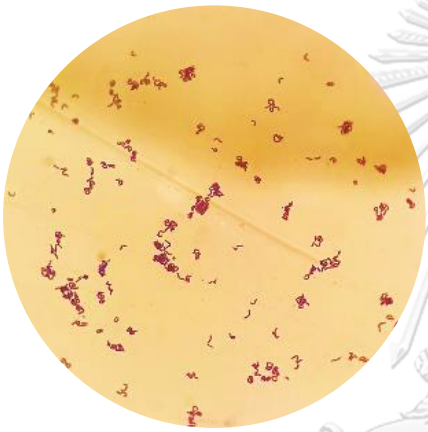
Figure: A2 Gram staining of isolated samples (continue)



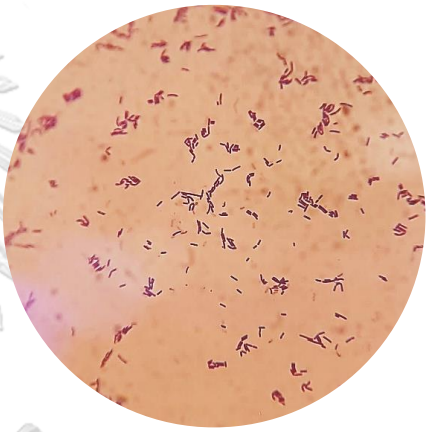
NYNE10.3



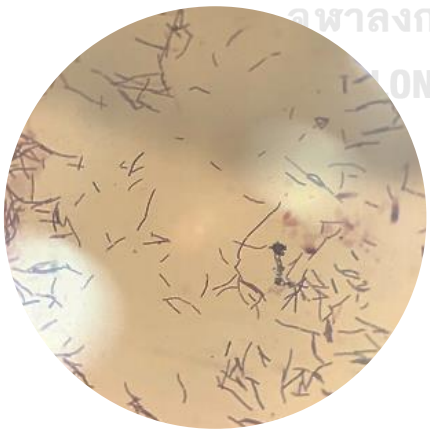
NYNE10.6



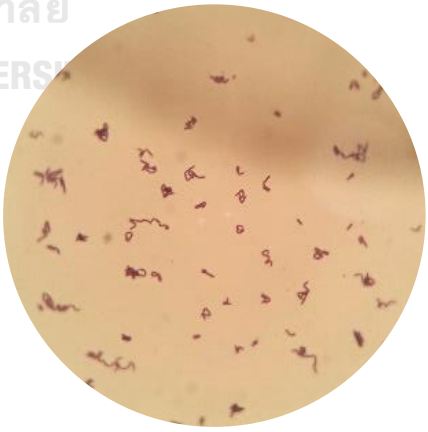
NYNE23.1



NYNS2S



NYNE24.5



NYNE23.5

Figure A2: Gram staining of isolated samples (continue)

Appendix-A3: Streaking isolates on MRS agar containing 0.3% $CaCO_3$

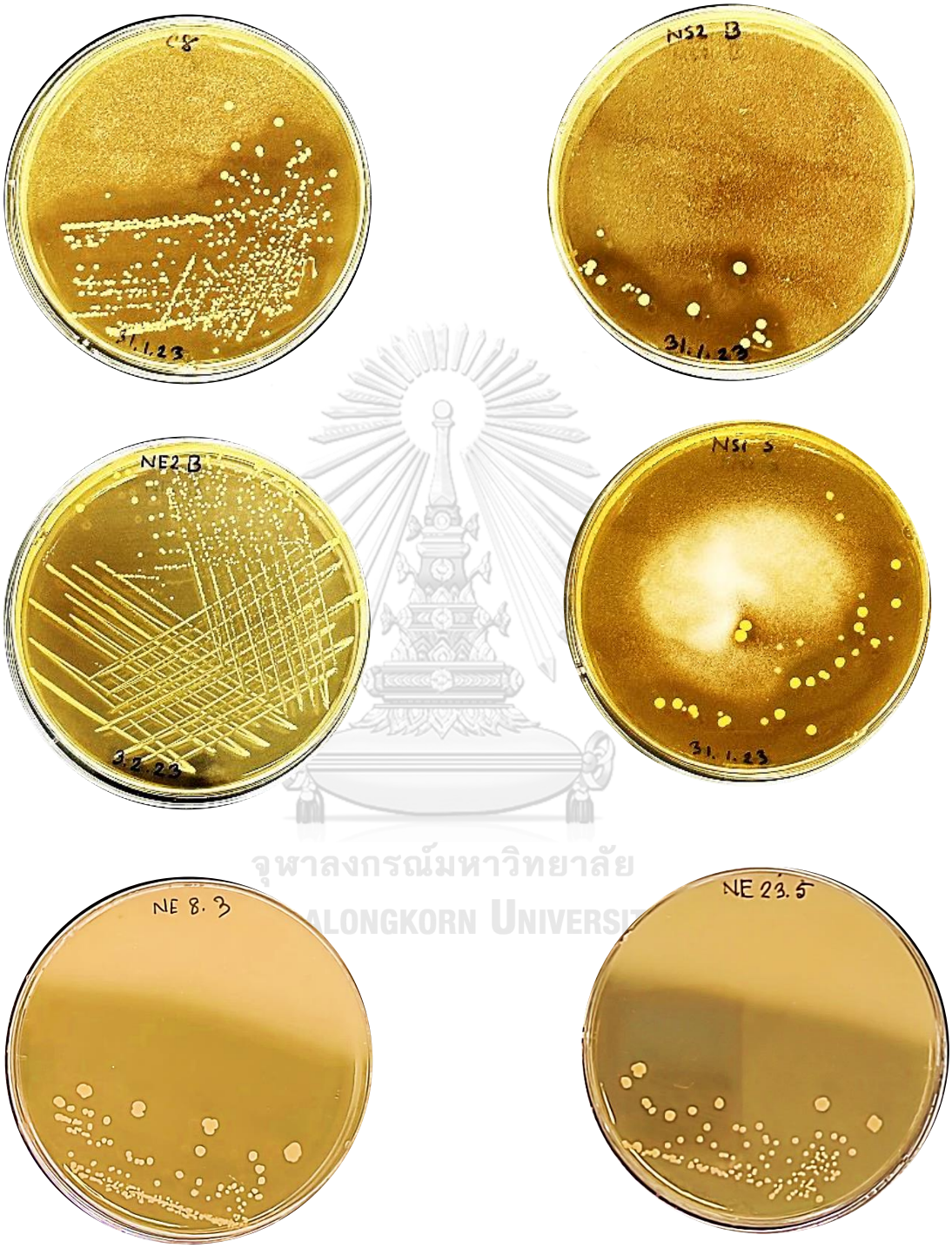


Figure A 3 *MRS agar containing 0.3% of $CaCO_3$*

Figure A3: MRS agar containing 0.3% of $CaCO_3$ (continue)

Figure A3: MRS agar containing 0.3% of $CaCO_3$ (continue)

Appendix -A4: L-cysteine standard curve preparation

L-cysteine standard curve initiated by constructing different concentrations (0.2, 0.4, 0.6, 0.8, 1 mM) of L-cysteine solution. Briefly, 0.5 ml of different concentrations L-cysteine was mixed with 1% of 0.5 ml potassium ferricyanide and 0.5 mL of phosphate buffer saline (PBS) at pH 6.6. The mixed solution was gone through subsequent incubation at 50 °C for 20 min and quick cooling. After cooling, 0.5 ml of 10% trichloroacetic acid (TCA) will be mixed and centrifuged for 5 min at 3000g to separate pellet and supernatant. 1 ml of separated supernatant was mixed with same volume of 0.1% ferric chloride and measured the absorbance at 700 nm after 10 min of reaction time. For the blank, L-cysteine was excluded, and same protocol was followed for rest steps. After subtracting blank absorbance from the absorbance of different concentration L-cysteine solutions, standard curve was constructed by placing the absorbance value against the concentration.

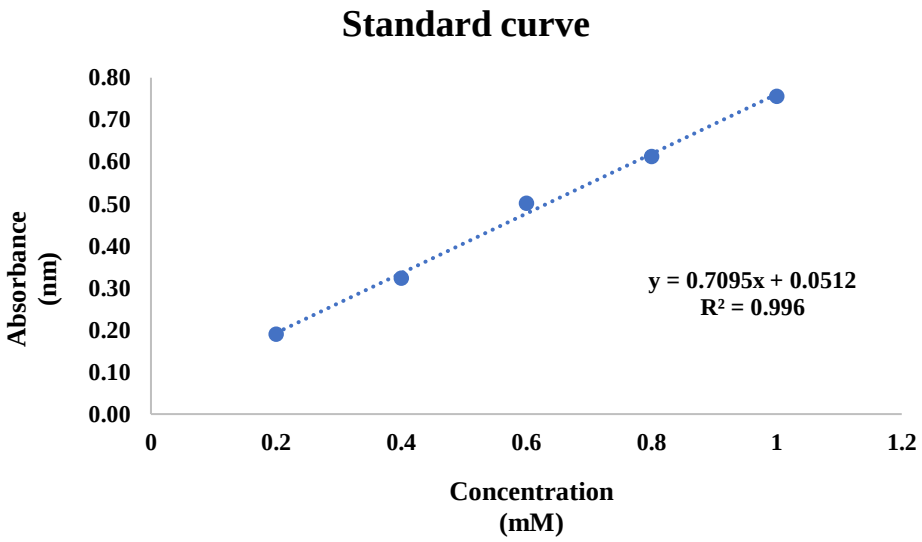


Figure A 4 Standard curve of L-cysteine

Table B 1 The ANOVA showing the interaction between the DPPH activity of supernatant and pellet of isolates

		Sum of Squares	df	Mean Square	F	Sig.
DPPH Supernatant	Between Groups	6221.329	17	365.961	6.710	.000
	Within Groups	1963.322	36	54.537		
	Total	8184.651	53			
DPPH Pellet	Between Groups	5394.076	17	317.299	12.280	.000
	Within Groups	930.214	36	25.839		
	Total	6324.29	53			

VITA

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PUBLICATION	1. Formulation and quality evaluation of instant soft drink powder prepared from hog plum (Spondius mangifera) and mint (Mentha spicata) 2. Effect of substituting wheat flour with buckwheat, honeyweed and stevia on nutritional and organoleptic properties of biscuit.