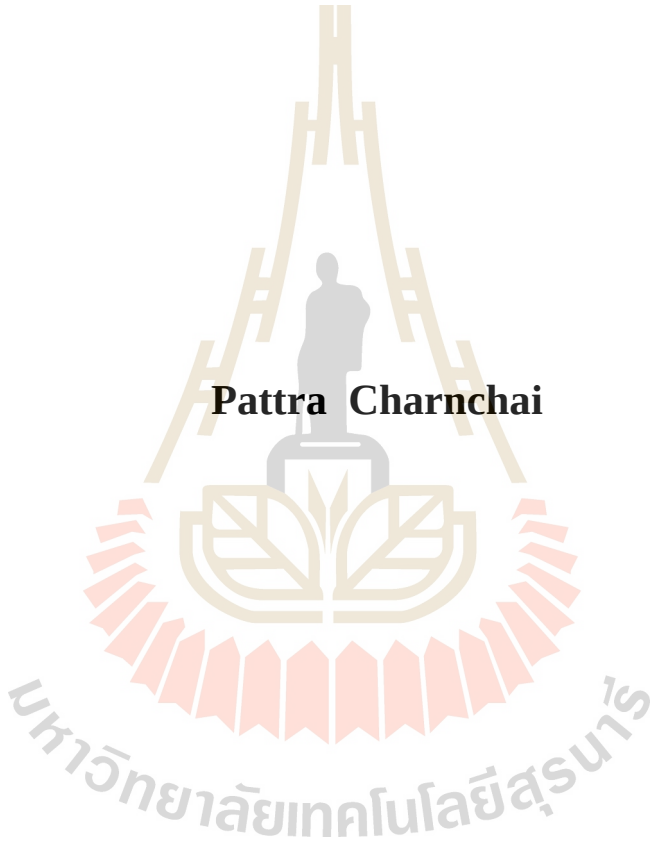
SELECTION AND CHARACTERIZATION OF *BIFIDOBACTERIUM* SPP. AND *LACTOBACILLUS* SPP. FOR PROBIOTIC STARTER

Pattra Charnchai



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การคัดเลือกและศึกษาสมบัติของ *Bifidobacterium* spp. และ *Lactobacillus* spp. เพื่อใช้เป็นกล้าเชื้อโพรไบโอติก

นางสาวภัทรา ชาญชัย

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต

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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

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ภัทรา ชาญชัย : การคัดเลือกและศึกษาสมบัติของ *Bifidobacterium* spp. และ *Lactobacillus* spp. เพื่อใช้เป็นกล้าเชื้อโพรไบโอติก (SELECTION AND CHARACTERIZATION OF *BIFIDOBACTERIUM* SPP. AND *LACTOBACILLUS* SPP. FOR PROBIOTIC STARTER) อาจารย์ที่ปรึกษา : รองศาสตราจารย์ ดร.เขมวิทย์ จันต๊ะมา, 165 หน้า.

กรรมวิธีในการผลิตในระดับอุตสาหกรรมอาจส่งผลกระทบต่อการมีชีวิตรอดและสมบัติในการทำหน้าที่เป็นโพรไบโอติก ส่งผลให้แบคทีเรียลดประสิทธิภาพในการทำหน้าที่ที่เป็นประโยชน์ต่อสุขภาพของมนุษย์ การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาสมบัติของแบคทีเรีย *Bifidobacterium* ที่คัดแยกได้จากอุจจาระของเด็กทารกไทยที่มีสุขภาพดี และ *Lactobacillus* ที่คัดแยกจากอาหารหมักดองท้องถิ่น ผลการศึกษาพบว่า *Bifidobacterium* จำนวน 3 สายพันธุ์ (BF014, BF052 และ BH053) และ lactobacilli จำนวน 4 สายพันธุ์ (LF005, LF022, LF026 และ LB013) มีความทนทานต่อสภาวะจำลองในระบบทางเดินอาหารได้เป็นอย่างดี โดย *Bifidobacterium animalis* BF052 แสดงสมบัติโพรไบโอติกได้ดีที่สุด เมื่อผ่านการทดสอบในระบบทางเดินอาหารจำลองพบว่าแบคทีเรียสายพันธุ์นี้มีความสามารถในการทนกรด และทนต่อน้ำย่อยจากน้ำดีได้ดี มีความสามารถในการยึดเกาะกับเซลล์เยื่อบุผนังลำไส้ Caco-2 ได้สูง และสามารถยับยั้งการเจริญของแบคทีเรียก่อโรค เช่น *Salmonella typhimurium* และ *Vibrio cholerae* ดังนั้นแบคทีเรียสายพันธุ์นี้จึงได้รับการคัดเลือกเพื่อนำมาทดสอบความสามารถในการมีชีวิตรอดและสมบัติของโพรไบโอติกเมื่อต้องผ่านกระบวนการผลิตแบบต่อเนื่อง อันได้แก่ กระบวนการทำแห้งแบบแช่เยือกแข็ง การเก็บรักษาผลิตภัณฑ์หลังจากทำแห้ง และความสามารถในการมีชีวิตรอดในผลิตภัณฑ์อาหารระหว่างการเก็บรักษา พบว่ากระบวนการผลิตไม่ได้ส่งผลกระทบต่อความคงตัวต่อสมบัติความเป็นโพรไบโอติกของแบคทีเรียสายพันธุ์นี้ โดยพิจารณาจากความสามารถในการทนต่อสภาวะจำลองในระบบทางเดินอาหาร และความสามารถในการยึดเกาะกับเซลล์ Caco-2 จึงแสดงให้เห็นว่า *B. animalis* BF052 ผ่านเกณฑ์อันน่าพอใจที่จะเป็นแบคทีเรียโพรไบโอติกที่ดีได้ และน่าจะใช้เป็นกล้าเชื้อโพรไบโอติกที่มีประสิทธิภาพในอุตสาหกรรมอาหารได้ และเพื่อพัฒนาอาหารที่ใช้เป็นตัวกลางในการขนส่งแบคทีเรีย *B. animalis* BF052 นั้น จึงได้ทดสอบพฤติกรรมของ *B. animalis* BF052 ในน้ำนมถั่วเหลือง และผลของการใช้จุลินทรีย์ตัวนี้ร่วมกับหัวเชื้อทางการค้า *Streptococcus thermophilus* และ *Lactobacillus bulgaricus* ระหว่างการหมักเป็นเวลา 48 ชั่วโมง ผลการทดลองพบว่า *B. animalis* BF052 ที่หมักร่วมกับเชื้อทางการค้านี้มีปริมาณเซลล์ที่มีชีวิตรอดจำนวนมาก และได้เนื้อสัมผัสของโยเกิร์ตที่มีความคงตัวที่ดีตลอดระยะเวลาการหมัก จากการทดสอบความพึงพอใจของผู้บริโภคพบว่าน้ำนมถั่วเหลืองที่หมักในช่วงเวลา 12-16 ชั่วโมง เป็นผลิตภัณฑ์ที่ผู้บริโภคพึงพอใจมากที่สุด

ทั้งลักษณะปรากฏ กลิ่น เนื้อสัมผัส รสชาติ และระดับการยอมรับโดยรวม ผลการทดลองแสดงให้เห็นว่า *B. animalis* BF052 สามารถใช้เสริมเป็นกล้าเชื้อโพรไบโอติกที่ใช้น้ำนมถั่วเหลืองเป็นสารตั้งต้นในการหมักได้ นอกจากนี้เพื่อให้เกิดความเข้าใจเชิงลึกในการปรับตัวตอบสนองของเชื้อต่อสภาวะแวดล้อมที่ไม่เหมาะสมและความสามารถในการใช้สารอาหารในระดับโมเลกุล จึงได้ตรวจวิเคราะห์ลำดับนิวคลีโอไทด์ทั้งจีโนมของ *B. animalis* BF052 พบว่าประกอบด้วยโครโมโซมแบบวงกลมที่มีจำนวนนิวคลีโอไทด์ทั้งหมด 1,938,624 คู่เบส ไม่มีพลาสมิด และได้เก็บข้อมูลนี้ไว้ในธนาคารจีโนมภายใต้เลขทะเบียน CP009045 ผลการวิเคราะห์ลำดับนิวคลีโอไทด์ทั้งจีโนมพบยีนที่มีความเกี่ยวข้องกับกลไกการปรับตัวหรือการตอบสนองของเชื้อเมื่อต้องอยู่ในสภาวะแวดล้อมที่ไม่เหมาะสม รวมทั้งยีนที่เกี่ยวข้องกับการใช้โอลิโกแซ็คคาไรด์ที่ไม่ถูกย่อยโดยเอนไซม์ในระบบทางเดินอาหารของเจ้าบ้าน

สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา_____

ปีการศึกษา 2559 ลายมือชื่ออาจารย์ที่ปรึกษา_____

PATTRA CHARNCHAI : SELECTION AND CHARACTERIZATION OF *BIFIDOBACTERIUM* SPP. AND *LACTOBACILLUS* SPP. FOR PROBIOTIC STARTER. THESIS ADVISOR : ASSOC. PROF. KAEMWICH JANTAMA, Ph.D., 165 PP.

PROBIOTICS/BIFIDOBACTERIA/FERMENTED SOYMILK/
WHOLE GENOME SEQUENCING

The viability and functionality of probiotics may be influenced by industrial production processes resulting in a decrease in probiotic efficiency that benefit the health of humans. This study aimed to investigate the probiotic characteristics of *Bifidobacterium* and *Lactobacillus* strains isolated locally from faecal samples of healthy Thai infants, and indigenous fermented foods, respectively. In the present work, three bifidobacterial strains (BF014, BF052, and BH053) and four lactobacilli strains (LF005, LF022, LF026 and LB013) showed a great resistance to conditions simulating the gastrointestinal tract. Among these, *Bifidobacterium animalis* BF052 possessed considerable probiotic properties, including high acid and bile tolerance through an *in vitro* model of gastrointestinal conditions, strong adhesion capability to Caco-2 cells, and inhibitory activity against pathogens including *Salmonella typhimurium* and *Vibrio cholerae*. This strain was thus selected as a promising probiotic strain to dertermine its viability and funtionality throughout food processing processes as well as the freeze-drying process, storage of freeze-dried powders, and incorporation of freeze-dried cells into food matrix on probiotic properties. The results demonstrated that the stability of the probiotic properties of *B. animalis* BF052 was not affected by the food processing chain, especially its resistance under the simulated

gastrointestinal conditions and its adherence ability to Caco-2 cells. It indicates that *B.animalis* BF052 satisfies the criteria as a potential probiotic and may be used as an effective probiotic starter in food applications. To develop a delivery medium for the live probiotic *B. animalis* BF052, the behavior of *B.animalis* BF052 and the effects of this organism as part of the starter cultures along with the conventional starters, *Streptococcus thermophilus* and *Lactobacillus bulgaricus* were investigated during the fermentation of soymilk for 48 h. It was observed that the behavior of *B. animalis* BF052 prepared with commercial yogurt starters showed high viable cell numbers and high consistency of yogurt texture throughout the fermentation period. Based on the consumers' preferences, soymilk fermented during 12 -16 h was the most preferable products in their overall preferences, including appearance, odor, texture, taste and overall acceptability. This result indicated that *B. animalis* BF052 could be supplemented as a probiotic starter that employs soymilk as the substrate. In addition, to gain insights into its adaptive responses to the environmental stresses and its capability to utilize specific substrates, the complete genome sequence of *B. animalis* BF052 was therefore determined. The *B. animalis* BF052 genome was composed of one circular chromosome of 1,938,624 bp with no plasmid and its sequence was deposited in GenBank under accession number CP009045. The screening of genome sequences revealed genes involved in adaptive responses to industrial and/or environmental stresses. Genes responsible for utilization of non-digestible oligosaccharides for intrinsic adaptation to the intestinal niche were also identified in *B. animalis* BF052 genome.

School of Biotechnology Student's Signature_____

Academic Year 2016 Advisor's Signature_____

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LIST OF ABBREVIATIONS

AhpC	=	Alkyl hydroperoxide reductase
ANOVA	=	One-way analysis of variance
BAs	=	Unconjugated bile acids
BHI	=	Brain-heart infusion agar
bp	=	Base pair
BS	=	Black sesame
BSHs	=	Bile-salt hydrolases
CFU/mL	=	Colony forming unit per milliter (s)
Clp	=	Caseinolytic proteases
CLSI	=	Clinical and laboratory standards institute
Csp	=	Cold-shock proteins
$^{\circ}$C	=	Degree celsius
DC	=	Dendritic cells
DMEM	=	Dulbecco's modified eagle's minimal essential medium
EDTA	=	Tris-ethylenediaminetetraacetic
F6PPK	=	Fructose-6-phosphate phosphoketolase
FAO	=	Food and agriculture organization
FD	=	Freeze-drying
FOS	=	Fructo-oligosaccharides
g	=	Gram (s)
g/L	=	Gram (s) per liter

LIST OF ABBREVIATIONS (Continued)

GadB	=	Glutamate decarboxylase
GadC	=	Glutamate/gamma-aminobutyrate antiporter
GBR	=	Germinated brown rice
GOS	=	Galacto-oligosaccharides
GRAS	=	Generally recognized as safe
h	=	Hour (s)
H_2O_2	=	Hydrogen peroxide
HPLC	=	High performance liquid chromatography
IEC	=	Epithelial cells
µL	=	Microliter (s)
µM	=	Micromolar (s)
mg	=	Milligram (s)
mg/L	=	Milligram (s) per liter
min	=	Minute (s)
mL	=	Milliliter (s)
mm	=	Millimeter (s)
mM	=	Millimolar
MRS	=	deMan, rogasa and sharpe
MutT	=	Triphosphate pyrophosphohydrolase
N	=	Newton
NADH	=	Reduced form of nicotinamide adenine dinucleotide
NAMs	=	N-acetylated metabolite

LIST OF ABBREVIATIONS (Continued)

NCBI	=	National center for biotechnology information
% (v/v)	=	Percentage volume by volume
% (w/v)	=	Percentage weight by volume
% (w/w)	=	Percentage weight by weight
PBS	=	Phosphate buffered saline
PCR	=	Polymerase chain reaction
PGAAP	=	Prokaryotic genomes automatic annotation pipeline
ROS	=	Reactive oxygen species
rpm	=	Revolutions per minute
SD	=	Standard deviation
SDS	=	Sodium dodecyl sulfate
SES	=	Sterile electrolyte solution
SOD	=	Superoxide dismutase
spp.	=	species
T	=	T-cells
TOS	=	Transgalacto-oligosaccharides
W_{fs}	=	Weight of the fermented soymilk
WHO	=	World health organization
WPC	=	Whey protein concentrate
W_s	=	Supernatant weight after centrifugation

CHAPTER I

INTRODUCTION

1.1 Significance of the study

Nowadays, consumers are more aware and concern about their lifestyle than ever before. This has an increased interest in the development of functional products that promote health and wellness. Such products and especially probiotics exert a beneficial effect on the balance of intestinal microbiota. Probiotics can be of benefit to the health of humans; however they must first be able to survive in sufficient numbers during manufacturing processes and storage as freeze-dried cultures, and also in the food products into which they are finally formulated. In addition, they should also possess the ability to survive in the gastrointestinal (GI) tract and retain their functionality to be effective in the host (Saarela et al., 2010). Consequently, probiotic strains selected for commercial applications must retain characteristics for which they were originally selected (Sharma et al., 2014). Recently, du Toit et al. (2013) demonstrated that the same probiotic strain presented different characteristics depending on the manufacturing and processing conditions.

In order to confer the health advantages, the level of probiotics in food products that serve as delivery systems needs to be high, suggesting minimum level of live probiotic cells should be at least 10^6-10^7 CFU/ml before consumption (Chaikham, 2005). The viability and stability of probiotics are challenges for industrial producers, and new technology has been developed to obtain highly stable probiotic starters with

their functionality. Consequently, a rigorous effort in strain selection and characterization should be regarded as a prerequisite (Solieri et al., 2014). This reinforces the need for robust probiotic bacteria that are able to survive stressful environmental challenges not only during industrial processes such as freeze-drying, manufacturing, and storage but also after consumption through GI tract stresses, until their adherence to the intestinal epithelium to exert health-promoting effects there (Ventura et al., 2007). Therefore, to guarantee a functional and effective probiotic strain with predictable health benefits, its viability and functionality throughout the food manufacturing processes and GI stress barriers must be investigated to ensure that health-promoting properties are maintained.

Besides the stability of probiotic during the mentioned processes, the fementation ability of the probiotic candidate is of prime importance. In this regard, the food matrix served as probiotic vehicle should specificially support probiotic growth during fermentation. It is generally know that probiotics products available in markets today are mainly milk based; however cultivation of probiotic bacteria in milk is a difficult task compared with that of conventional starters. Nowadays, the demand for alternatives to dairy products is growing due to problems with lactose intolerance, cholesterol content, allergenic milk proteins and desire for vegetarian alternatives (Santos et al., 2014). Among the non-dairy probiotic products, soymilk based yogurt is especially attractive because it is rich in health-beneficial substances with high nutritive values (Ma et al., 2015). It was also reported that soymilk contains available carbohydrates that could be fermented by most of the strains belonging to lactic acid bacteria and bifidobacteria (Sumarna, 2008). Therefore, it is a great challenge to develop soymilk as a delivery medium for the candidate probiotic which

aims to transfer probiotic health benefits and nutritional substances of soymilk to the consumers. It is also anticipated that soymilk supplemented with selected probiotic would offer not only a means of enhancing beneficial health properties but also a possibility for modifying or improving its flavor and texture. The understanding of the roles of a promising probitic candiate as a technological starter would be thus useful in developing suitable food matrices specifically benefit to the consumers.

The lack of knowledge regarding the molecular or genetic basis of proposed probiotic benefits greatly weakens the scientific credibility of health claims (Lee and O'Sullivan, 2010). With this regard, the explosion in the availability of genome sequences can provide a means to fulfill the gap of knowledge. Therefore, it is no doubt to determine a whole genome sequencing of a certain probiotic. The genetic data can be translated into biologically relevant information by an interactive combination of bioinformatics and experimental approaches. This will provide valuable sources in developing and enhancing probiotic efficiency for potential use in commercially important applications.

1.2 Objectives

1) To investigate the probiotic characteristics of *Bifidobacterium* spp. and *Lactobacillus* spp. isolated from faecal samples of healthy Thai infants and traditional fermented foods, respectively, as a highly stable probiotic starter. The candidate strains were initially screened on the basis of acid and simulated gastric tolerance and were further screened for functional properties, such as antimicrobial activity and adhesion ability. In addition, the impact of the production process chain on the selected probiotic's survival and resistance to GI stress, and its adhesion ability to

Caco-2 cells were also investigated. This part of the study was to ensure that the strain would still provide probiotic effects after consumption.

2) To investigate the behavior of the probiotic *B.animalis* BF052 in soymilk and the effects of this organism as part of the starter cultures along with the commercial starters, *S. thermophilus* and *L. bulgaricus*. The sensory preferences of fermented soymilk prepared in combination with those starters were also evaluated. This work demonstrated the possibility in applying *B. animalis* BF052 as a probiotic starter in the production of fermented milk.

3) To reveal genetic features of the probiotic *B. animalis* BF052 from genome sequence data. This work provided genes involved in adaptive responses to industrial and/or environmental stresses and in utilization of specific carbohydrates.

CHAPTER II

LITERATURE REVIEW

2.1 Definition of probiotics

The concept of probiotics has been around for more than a century and products containing probiotic organisms are one of the largest growing markets, representing 60-70% of the total functional food markets (Sharma et al., 2014). The universal meaning of the term "probiotic" was established by the World Health Organization (WHO) and the Food and Agriculture Organization (FAO) of the United States by defining as "live microorganisms which when administered in adequate amounts confer a health benefit on the host organism" (FAO/WHO, 2006). Élie Metchnikoff is considered to be the inventor of probiotics. Intrigued by the longevity of the Caucasian population and its frequent consumption of fermented milks, Metchnikoff proposed that the acid-producing organisms in fermented dairy products could prevent "fouling" in the large intestine and thus lead to a prolongation of the life span of the consumer. Different microorganisms have been used thereafter as probiotics in the last century for their ability to prevent and cure diseases (Heller, 2001). Nowadays, there is solid scientific evidence to support the concept that these bacteria transit GI tract and help to maintain or create a favorable microbial conditions to provide healthy digestive function and confer therapeutic benefits for the consumer. Many studies have also demonstrated the efficiency of probiotics at offering an appropriate alternative to the use of antibiotics in the treatment of enteric infection or

to reduce the symptoms of antibiotic-associated diarrhea (Giraffa et al., 2010).

2.2 Criteria for the selection of probiotics

In the development of probiotic foods intended for human consumption, viability during processing operations and storage, survival during intestinal transit and potential health benefits on consumers are the primary criteria for selecting suitable strains of probiotic applications. In addition, the selected probiotic strains should be suitable for large-scale industrial production with the ability to survive and retain their functionality during production and storage as frozen or dried cultures. They must also survive in the food products into which they are finally formulated as well as exhibiting the compatibility with the conventional starters while added to the end product. For some positive effects on human health, probiotic strains have to reach the large intestine at a recommended level. In addition, these strains should be metabolically active within GI tract and biologically effective against the identified target. Therefore, to have functional probiotic strains with predictable and measurable health benefits, a rigorous effort for strain selection is required. Numerous criteria have been recognized and suggested for selection of suitable probiotic organisms (Sharma et al., 2014; Tripathi and Giri, 2014; Ventura et al., 2007). Table 2.1 revealed criteria for selection of probiotics in commercial applications. However, it is important to note that each such strain has its own specific properties and ideal strains must have established health and safety data from randomized, controlled clinical trials (Sharma et al., 2014).

Table 2.1 Criteria for the selection of probiotics in commercial applications.

General criteria	Property
Safety criteria	- Pathogenicity and infectivity - Origin - Virulence factors (nonpathogenic, nontoxic, nonallergic, nonmutagenic and not carry transmissible antibiotic resistance)
Technological criteria	- Genetically stable strains - High viability during processing and storage - Good sensory properties - Large-scale production - Phage resistance
Functional criteria	- Tolerance to gastric acid - Bile tolerance - Adhesion to mucosal surface - Validated and documented health effects
Physiological criteria	- Antagonistic activity towards GI pathogens - Cholesterol metabolism - Lactose metabolism - Immunomodulation - Antimutagenic and anticarcinogenic properties

2.3 Microorganisms used as probiotics

Though a wide variety of genera and species of microorganisms are considered as potential probiotics, the one used commercially in probiotic foods are predominantly bacteria from the genera *Bifidobacterium* and *Lactobacillus*. The primary reason being both of these genera have a long history of safe use and are considered as GRAS (generally recognized as safe) (Tripathi and Giri, 2014). However, species belonging to the genera *Lactococcus*, *Enterococcus*,

Saccharomyces and *Propionibacterium* are also considered as probiotic due to their health-promoting effects. Nowadays, multistrains or multispecies probiotic mixtures are becoming increasingly popular compared with single strain probiotics as they may have additive or even synergistic effects which can result in higher efficacy (Chapman et al., 2011). Table 2.2 lists the example of commercial probiotic microorganism used today (Shah, 2007).

2.3.1 *Bifidobacterium*

Bifidobacteria belong to the *Bifidobacterium* genus which includes over 40 proposed *Bifidobacterium* species, some of which are commonly recovered from the intestinal tract of humans and animals. *Bifidobacterium* spp. was first isolated from the faeces of breast-fed infants by Henry Tissier in 1899. They belong to the dominant microbiota (>1%) in adults and can represent up to 90% of the faecal anaerobic bacteria of breast-fed infants (Lee and O'Sullivan, 2010). In full term neonates, bifidobacteria are among the first strictly anaerobic bacteria colonizing the gut during the first week of life. In premature neonates, bifidobacteria gut colonization has been reported to be delayed leading to a dysbiosis with potential health consequences. More than one century ago Tissier suggested that the large number of bifidobacteria recovered from faeces of healthy breast-fed infants was likely the reason for their low incidence of infantile diarrhea. Because bifidobacteria may contribute to the maintenance of GI health and due to their potential beneficial effects on their host, they are currently used as health-promoting agents in a large variety of dietary supplements and functional food products (Prasanna et al., 2014).

Table 2.2 Commercial probiotic microorganisms.

Microorganism	Strain	Company (product)
Bifidobacterium animalis	BB-12	Chr. Hansen
Bifidobacterium bifidum	BB-11	Chr. Hansen
Bifidobacterium essencis	-	Danone (Activia)
Bifidobacterium lactis	Bb-02, Lafti™ B94 HN019(DR10™)	DSM Danisco
Bifidobacterium longum	BB536 SBT-2928 UCC 35624	Morinaga Milk Industry Snow Brand Milk Products UCC 35624
Bifidobacterium breve	-	Yakult
Lactobacillus acidophilus	LA-1/LA-5 NCFM DDS-1 SBT-2062 La1	Chr. Hansen Rhodia Nebraska Cultures Snow Brand Milk Products Nestle
Lactobacillus casei	Shirota Immunitas	Yakult Danone
Lactobacillus fermentum	RC-14	Urex Biotech
Lactobacillus lactis	L1A	Essum AB
Lactobacillus paracasei	CRL 431	Chr. Hansen
Lactobacillus rhamnosus	GG GR-1 LB21 271	Valio Urex Biotech Essum AB Probi AB
Lactobacillus plantarum	299v Lp01	Probi AB
Lactobacillus reuteri	SD2112/ MM2	Biogaia

Bifidobacteria are non-motile, non-spore-forming, non-gas-producing, Gram-positive, anaerobic, catalase-negative bacteria with a high G+C content (55 to 67%). Their morphology is generally referred to as bifid or irregular V- or Y-shaped rods resembling branches. The actual reason for the irregular shape of bifidobacteria is not yet clearly understood. Under unfavourable conditions bifidobacteria show

branching and pleomorphism, although they are predominantly rod shaped in their natural habitat. It has been demonstrated that the absence or low concentrations of N-acetylglucosamine, amino acids (alanine, aspartic acid, glutamic acid and, serine), and Ca^{2+} ions in the growth medium exclusively induce the bifid shape of bifidobacteria (De Dea Lindner et al., 2007; Lee and O'Sullivan, 2010).

In general, bifidobacteria metabolize hexoses using the "bifidus pathway". The key enzyme involved in this pathway is fructose-6-phosphate phosphoketolase (F6PPK), which is present intracellularly. The F6PPK catalyses the splitting of fructose-6-phosphate to erythrose-4-phosphate and acetyl-phosphate. Hexoses such as glucose or fructose are metabolized via this pathway to acetate and lactate in a theoretical ratio of 3:2 for energy production. This enzyme also serves as a taxonomic tool in the identification of the genus but does not allow the distinction at the species level. The optimum temperature for the growth of bifidobacteria is 37-41°C. Most *Bifidobacterium* strains originating from humans have been reported to grow optimally at a temperature of 36-38°C, whereas animal strains grow optimally at 41-43°C. The optimum pH for the growth of bifidobacteria is 6-7. However, certain strains of *B. lactis* and *B. animalis* were shown to grow even at pH 3.5 (Lee and O'Sullivan, 2010; Prasanna et al., 2014).

Bifidobacteria are considered to provide many beneficial effects including improvement of lactose digestibility, anticarcinogenic activity, reduction of serum cholesterol level, synthesis of B vitamins and facilitation in calcium absorption. Even though *Bifidobacterium* strains are already used in dairy products, they have some inferior characteristics compared to the traditional lactic acid bacteria used in fermented dairy products, which hinder their possible applications. More specifically,

they have weaker growth and acid production in cows' milk and require long fermentation times, anaerobic conditions, and low redox potential for their growth. During their growth, bifidobacteria generally produce acetic acid and lactic acids, however, they are unable to generate sufficient amounts of lactic acid for the manufacture of fermented milk products with the appropriate aroma and flavour. Therefore, they are often incorporated as co-cultures into fermented dairy products, such as yogurt which are fermented by ordinary lactic acid bacterial starters, such as *S. thermophilus* and *L. delbrueckii* ssp.bulgaricus (Lee and O'Sullivan, 2010; Prasanna et al., 2014; Sharma et al., 2014).

2.3.2 *Lactobacillus*

The genus *Lactobacillus* belonging to the large group of lactic acid bacteria which are Gram-positive, non-spore-forming rods, catalase-negative, aero-tolerant or anaerobic, acidophilic and are nutritionally fastidious. They generally have a fermentative metabolism, whose primary fermentation end product is lactic acid, besides other products such as acetate, ethanol, CO_2, formate and succinate. Because the main catabolite is lactic acid, lactobacilli prefer relatively acidic conditions (pH 5.5-6.5) (Giraffa et al., 2010).

The genus *Lactobacillus* can be found in a variety of ecological niches such as plants, animals and raw milk. The ability to colonize such a variety of habitats is a direct consequence of the wide metabolic versatility of this group of lactic acid bacteria. Numerous species of *Lactobacillus* are relevant in fermented foods as they have been used as starter and/or protective cultures in fermented vegetables, dairy products, sausages and fish. These microorganisms are considered as generally

regarded as safe (GRAS) due to their long history of use as food processing aids, and some strains of *Lactobacillus* were shown to confer a health benefit on humans and animals (Abriouel et al., 2015). The extensively studied probiotics are *Lactobacillus* strains, whose contribution to counteracting the effect of a wide range of infections, such as antibiotic-associated diarrhea, *Helicobacter pylori* gastroenteritis and urovaginal infections have been demonstrated in both *in vitro* and *in vivo* experimental studies as well as in clinical trials. Some of the identified antimicrobial compounds produced by *Lactobacillus* strains include organic acid, hydrogen peroxide, diacetyl, reuterin and bacteriocins. Because of their potential therapeutic and prophylactic attributes, lactobacilli have also been proposed as probiotics (Giraffa et al., 2010).

2.4 Food matrices to deliver probiotics

Consumer behavior towards food choice is changing due to the profound understanding in the relationship between diet and health. Fermented beverages with probiotic bacteria are very important to the human diet around the world because fermentation is an economical technology that helps preserve the food, improve its nutritional values and enhance its sensory properties. The application of probiotic cultures in different food matrices (dairy and non-dairy based beverages), could represent a great challenge for delivering viable probiotics to the consumer.

2.4.1 Dairy probiotic products

In dairy applications, probiotics are delivered with different fermented dairy products, most notable yogurt. In addition to exceptional nutritional attributes, milk and milk-derived products such as fermented milk contain components that

possess a range of different bioactivities. Dairy starter cultures and some probiotics have appreciable proteolytic activity, which is required for their rapid growth in milk. During fermentation, milk proteins, namely caseins, undergo a slight proteolytic degradation resulting in a number of potentially bioactive peptides. Lactic acid bacteria and their metabolites play a key role in enhancing microbiological quality and shelf life of fermented dairy products. The type of microorganisms used in the fermentation (single culture or mixed cultures) is an important factor, which determines the nutritional and sensory properties of the fermented dairy products (Prasanna et al., 2014). *Lactobacillus* and *Bifidobacterium* are the bacteria that are most frequently used as probiotics. These bacteria grow slowly in milk because they lack essential proteolytic activity and for this reason they are usually combined with *S. thermophilus*. Thus, the use of these combinations allows dairy processors to produce fermented dairy products with the desired technological characteristics, as well as with potential nutritional and health benefits (Casarotti et al., 2014). In addition, the expansion of the dairy industry leads to the introduction of hybrid dairy products, made by combining the dairy and fruit drink markets to offer healthier, more convenient and more flavorful products. Fermented milk enriched with fruit is responsible for more than half of this market segment of the dairy industry. Prepared fruits, in the form of pieces, pulp and even flour prepared from processing the peel, have been successfully incorporated with probiotic yogurts as sources of prebiotic fibers and nutrients that stimulate the growth and activity of intestinal microbiota (Martin et al., 2013; Shori et al., 2016). Other vehicles that could be used to deliver probiotics are ice cream and frozen dairy desserts. These products have the advantage

to be stored at low temperatures, which makes them less exposed to abusive temperatures having higher viability at the time of consumption.

2.4.2 Nondairy probiotic products

Nowadays, nondairy probiotic products have a big worldwide importance due to the ongoing trend of vegetarianism and to a high prevalence of lactose intolerance in many populations around the world. This fact has led to the launch of new products based on non-dairy matrices. Some matrices have been used in the development of non-dairy probiotic products such as fruits, vegetables, legumes and cereals. Fruits and vegetables can be considered good matrices since they contain nutrients such as minerals, vitamins, dietary fibres, and antioxidants, while lacking the dairy allergens that might prevent consumption by certain segments of the population (Sheehan et al., 2007). Currently, commercial products include fermented and unfermented fruits and vegetables, organic probiotic drinks and dried fruit enriched with probiotic microorganisms. These products have a healthy appeal, which attracts consumers. However, the incorporation of probiotics in fruit juices requires the protection against acid conditions. This can be achieved by microencapsulation technologies, which allow the entrapment of cells into matrices with a protective coating. Previous studies identified appropriate probiotic strains for incorporation into vegetable juices (Table 2.3).

Probiotic strains usually found in vegetable materials are species belonging to *Lactobacillus* and *Leuconostoc* genera. The strains *L. plantarum*, *L. casei* and *L. delbrueckii*, for example, were able to grow in cabbage juice without nutrient supplementation and reached 10^8 CFU/mL after 48 h of incubation at 30°C

(Yoon et al., 2006). In addition, it was found that those bacteria also grew in beet juice (Yoon et al., 2005).

Table 2.3 Application of selected probiotic bacteria in fermented vegetable based beverages.

Beverage	**Probiotic bacteria**	**References**
Beet juice	*L. acidophilus, L. plantarum,* *L. casei* and *L. delbrueckii*	Yoon et al. (2005)
Tomato juice	*L. acidophilus* LA39, *L. plantarum* C3, *L. casei* A4 and *L. delbrueckii* D7	Yoon et al. (2004)
Carrot juice	*L. bulgaricus, L. paracasei* Lpc-37, *L. rhamnosus* GG, *L. plantarum* Lp-115 and *B. lactis* 420,	Kun et al. (2008), Nazzaro et al. (2008), Tamminen et al. (2013)
Cabbage juice	*L. brevis, L. rhamnosus,* *L. plantarum* C3, *L. casei* A4 and *L. delbrueckii* D7	Jaiswal and Abu-Ghannam (2013), Yoon et al. (2006)
Green tea	*L. paracasei* LAFTI-L26, *L. acidophilus* LAFTI-L10 and *B. lactis* LAFTI-B94	López de Lacey et al. (2014)
Herbal mate	*L. acidophilus* ATTC 4356	Lima et al. (2012)
Soymilk with inulin and okara flour	*L. acidophilus* La-5, *B. lactis* BB-12 and *S. thermophilus*	Bedani et al. (2013)
Soymilk	*L. acidophilus* L10, *B. lactis* B94 and *L. casei* L26	Donkor et al. (2007)

In the case of cereals, the fermentation with probiotic microorganisms could be beneficial due to the decrease of nondigestible carbohydrates (poly- and oligo-saccharides), the improvement of the quality and level of lysine, the availability of the vitamin B group, as well as the degradation of phytates and release of minerals (such as manganese, iron, zinc, and calcium) (Blandino et al., 2003). Malt, wheat and barley extracts demonstrated to have a good influence in increasing bile tolerance and viability *of L. acidophilus*, *L. reuteri* and *L. plantarum* (Patel et al., 2004; Michida et al., 2006).

2.4.3 Soy-based probiotic products

Soybean is the most important legume in the traditional oriental countries' diet, which can provide inexpensive high-quality proteins and essential amino acids. Apart from proteins, soybean contains basic nutritive constituents, such as lipids, vitamins, minerals, free sugar and contains isoflavones, flavanoids, saponins and peptides that are of therapeutic value. Isoflavones have been linked to reduced risk of most hormone-associated health disorders. Soy-based foods may also provide a range of health benefits to consumers due to their hypolipidemic, anticholesterolemic and antiatherogenic properties as well as reducing in allergenicity (Trindade et al., 2001). Fermentation of soybean gives rise to different products based on many criteria but microbes are the foremost cause for the differences as they affect the aroma, texture, therapeutical and neutraceutical values (Sanjukta and Rai, 2016). However, consumption of soybean milk is hindered due to the presence of unpleasant off-flavors carried over from soybeans. These characteristic flavors are caused by n-hexanal and pentanal, which occur in beans as a product of breakdown of unsaturated

fatty acids (Scalabrini et al., 1998). In addition to these aldehydes, soymilk contains various oligosaccharides including raffinose and stachyose that may cause a GI discomfort to consumers. Raffinose and stachyose are α-galactosides of sucrose and are non-digestible in the gut due to the absence of α-galactosidase in the human intestine. Consequently, intact oligosaccharides pass directly into the lower intestine where they are metabolized by bacteria that possess this enzyme, resulting in the production of gases. Fermentation has been a traditional option to increase digestibility of soy products and make them more flavored. It was reported that soymilk can be a good culture medium for inoculation and growth of probiotic strains (Wang et al., 2003). The genus *Bifidobacterium* and *Lactobacillus* have α-galactosidase activity, which enables them to utilize sugars such as raffinose and stachyose, and sufficient proteolytic activity to support growth in soymilk (Farnworth et al., 2007). Many studied reported the ability of the strains to ferment soymilk including *B. longum*, *B. breve* and *B. infantis* (Hou et al., 2000; Garro et al., 2004; Scalabrini et al., 1998), *L. helveticus* (Murti et al., 1993), *L. fermenti* (Chumchuere and Robinson, 1999), *L. fermentum* (Garro et al., 2004), *L. reuteri* (Tzortzis et al., 2004), and *L. acidophilus* (Wang et al., 2002, 2006). This has led to the designing of the probiotic soy yogurt.

According to Euromonitor (2008), the market of probiotic soy yogurts represented 12% of the total sales of soy-based products in 2005, with an increase of 17% annually. In addition, researches have shown that probiotic soy-based products in combination with fruit juices are successful in the maintenance of both probiotic and sensory properties. A higher demand of these products indicates that consumers have incorporated them into their regular diet, changing their attitude toward soy and

its by-products, and also changing their expectations with regard to new probiotic soy-based products available in the marketplace.

2.5 Mechanism of probiotics

The modes of action by which probiotics are thought to contribute to human health fall into three main categories (Lebeer et al., 2010). First, certain probiotics can exclude or inhibit pathogens. This is currently the best studied probiotic mechanism and has been exhaustively reviewed elsewhere. A second mechanism is to enhance the function of the intestinal epithelial barrier by modulating the various signalling pathways that lead to, for example, the induction of mucus and defensin production, enhancement of tight junction functioning and prevention of apoptosis. The third method is to modulate host immune responses, resulting in both local and systemic. Figure 2.1 shows a schematic overview of the potential mechanisms whereby probiotic microorganisms might influence the intestinal microbiota (O'Toole and Cooney, 2008).

Consumption of probiotic cultures may modulate the microbiota or change its metabolic properties by competition for nutritional substrates. Sonnenburg et al. (2006) used transcriptional microarrays to show that introducing a probiotic into the mouse gut changes the way the endogenous microbiota metabolize the diet. Thus, one of the ways in which probiotics can impact upon the composition of the microbiota is apparently by competing with them for substrate availability and by altering the dynamics of carbohydrate utilization by individual microbiota components. This competition is probably not restricted to the intestine, since recent evidence indicates that oral *Bifidobacterium* strains reduce vitamin K concentration, and may thus compete with *Porphyromonas gingivalis* in the oral cavity (Hojo et al., 2007).

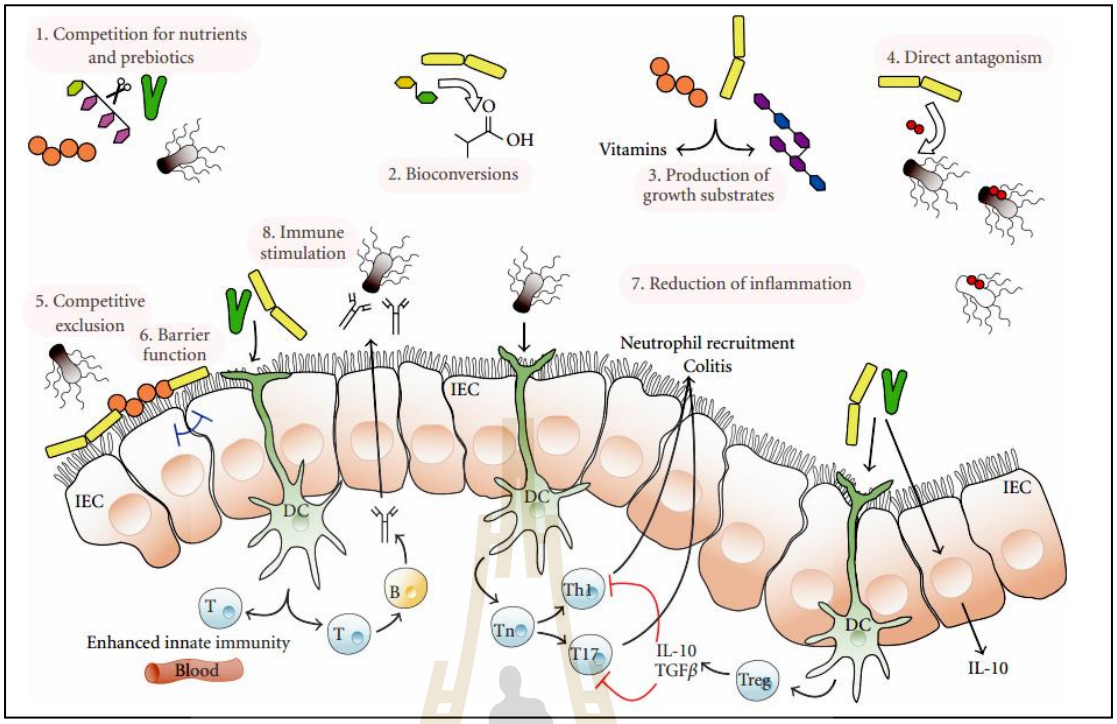


Figure 2.1 Schematic diagram illustrates potential or known mechanisms whereby probiotic bacteria impact on the host health. These mechanisms include (1) competition for dietary ingredients as growth substrates, (2) bioconversion of, for example, sugars into fermentation products with inhibitory properties, (3) production of growth substrates, for example, EPS or vitamins, for other bacteria, (4) direct antagonism by bacteriocins, (5) competitive exclusion for bindingsites, (6) improved barrier function, (7) reduction of inflammation, thus altering intestinal properties for colonization and persistence within, and (8) stimulation of innate immune response (by unknown mechanisms). IEC: epithelial cells, DC: dendritic cells, T:T-cells (O'Toole and Cooney, 2008).

The application of metabolic profiling methods to animal models has suggested another indirect way in which probiotic bacteria might impact on the microbiota, namely, by production of a significantly different microenvironment due to a diverse range of metabolic pathway outcomes. Martin et al. (2004) observed microbiome modification in germ-free mice colonized by human baby microbiota and exposed to two lactobacilli strains. This was accompanied by changes in cecal concentrations of short-chain fatty acids, and marked changes in faecal levels of

diverse metabolites including choline, acetate, ethanol, a range of putative N-acetylated metabolites (NAMs), unconjugated bile acids (BAs), and tauro-conjugated bile acids. Fukuda et al. (2011) proposed that the production of acetate by *B. longum* subsp. longum JCM1217, *B. longum* subsp. infantis 157F, and *B. longum* subsp. longum NCC 2705 was able to protect mice against death induced by enterohaemorrhagic *Escherichia coli* O157:H7. It is likely that such gross changes in metabolic profile also impact upon intestinal microbiota composition. In addition, exopolysaccharide produced by probiotics including lactic acid bacteria act as a growth substrate for selected components of the microbiota (Bello et al., 2001).

Probiotic bacteria probably also impact on the general microbiota by direct antagonism. Natural competition between commensals and opportunistic pathogens may therefore be mediated by mechanisms such as bacteriocin production, that can be exploited for using probiotics to modulate the microbiota. The production of bacteriocin Abp118 by intestinal *L. salivarius* was identified as the mechanism whereby *L. salivarius* UCC118 eliminated *Listeria monocytogenes* infection in a murine model, providing the first definitive mechanism for anti-infective activity of a probiotic bacterium in *vivo* (Barrett et al., 2007; Corr et al., 2007). Competitive exclusion, whereby adherent probiotic species occlude access of members of the microbiota to the epithelium (collado et al., 2008), represents another way of modulating the microbiota, although strong evidence for this occurring *in vivo* is lacking.

The most subtle effects wrought by probiotics on the microbiota are potentially those that operate by indirect mechanisms involving the host. It is well established that some probiotics can suppress inflammation by inhibiting

proinflammatory cytokine production (O'Hara et al., 2006). Reduction in gut inflammation by probiotics could plausibly alter the gut environment sufficiently to impact on the microbiota. Cell surface macromolecules (such as long surface appendages, polysaccharides and lipoteichoic acids) are key factors in this beneficial microorganism-host crosstalk, as they can interact with host pattern recognition receptors (PRRs) of the GI mucosa, resulting in probiotic effects (Lebeer et al., 2010). Some probiotic bacteria have been reported to stimulate the innate immune system both in animal models and in elderly subjects (Gill et al., 2000). Administration of probiotic bacteria could thus bolster innate immune activity against transient pathogens, or non-commensal elements in the microbiota, leading to subtle changes in long-term overall composition.

2.6 Health effects of probiotics

A number of health benefits are claimed in favour of products containing probiotic organisms including antimicrobial activity and GI infections, improvement in lactose metabolism, antimutagenic properties, anticarcinogenic properties, reduction in serum cholesterol, anti-diarrhoeal properties, immune system stimulation, improvement in inflammatory bowel disease and suppression of *Helicobacter pylori* infection (Table 2.4). Primary clinical interest in the application of probiotics has been in the prevention and/or treatment of infectious diseases including bacterial- and viral-associated diarrhea. The use of probiotics for control of chronic inflammatory diseases such as pouchitis and ulcerative colitis has also received considerable attention.

Table 2.4 Some of the established and potential health benefits of probiotic organisms (Vasiljevic and Shah, 2008).

Health effect	Mechanism
Alleviation of lactose intolerance	Delivery of intracellular β-galactosidase into human GI tract
Prevention and reduction of symptoms of rotavirus and antibiotic associated diarrhoea	Competitive exclusion Translocation/barrier effect Improved immune response
Treatment and prevention of allergy (atopic eczema, food allergy)	Translocation/barrier effect Immune exclusion, elimination and regulation
Reduction of risk associated with mutagenicity and carcinogenicity	Metabolism of mutagens Alteration of intestinal microecology Alteration of intestinal metabolic activity Normalization of intestinal permeability Enhanced intestinal immunity
Hypocholesterolemic effect	Deconjugation of bile salts
Inhibition of *H. pylori* and intestinal pathogens	Competitive exclusion Barrier effect Production of antimicrobial compounds
Prevention of inflammatory bowel diseases	Competitive exclusion Improvement of epithelial tight junctions Modification of intestinal permeability Modulation of immune response Production of antimicrobial products Decomposition of pathogenic antigens
Stimulation of immune system	Recognition by toll-like receptors induction of innate and adaptive immunity: -Downregulation of pro-inflammatory cytokines and chemokines -Upregulation of phagocytic activity -Regulation of Th1/Th2 balance

Recently, there is growing interest in using probiotics to reduce health risks, including the development of dental caries, allergy or even cancer. Some of the health benefits are well established, while other benefits have shown promising results in

animal models. However, additional studies are required in humans to substantiate these claims. Health benefits imparted by probiotic bacteria are strain specific, and not species- or genus specific. It is important to note that no strain will provide all proposed benefits, not even strains of the same species, and not all strains of the same species will be effective against defined health conditions. (Marco et al., 2006; Shah, 2007; Vasiljevic and Shah, 2008).

2.7 Factors influence probiotic viability

Many factors were found to influence the viability of probiotic microorganisms in food products during production, processing and storage. The identified factors include food parameters (pH, titratable acidity, molecular oxygen, water activity, presence of salt, sugar and chemicals like hydrogen peroxide, bacteriocins, artificial flavoring and coloring agents); processing parameters (heat treatment, incubation temperature, cooling rate of the product packaging materials and storage methods, and scale of production); and microbiological parameters (strains of probiotics, rate and proportion of inoculation). The viability and activity of probiotic cultures may be affected during steps involved in a delivery process through the exposure to different stress factors (Table 2.5) (Shori et al., 2016; Tripathi and Giri, 2014). In addition, the composition of the food, types of packaging material and storage environment (storage temperature, moisture content of powders, relative humidity, oxygen content, and exposure to light, among others) also have significant influences on the survival of probiotics.

Table 2.5 Different stress vectors affecting viability of probiotic during processing.

Processing step	Stress vector
Production of probiotic preparations	- Fermentation conditions; Fermentation medium, pH and acidity, temperature and dissolved oxygen - Presence of organic acids during cultivation - Concentration; high osmotic pressure, low water activity, higher concentration of particular ions - Temperature; freezing, vacuum and spray drying - Drying/Protective agents - Food ingradients - Packaging and storage conditions - Prolonged storage; oxygen exposure
Production of a probiotic containing product	- Nutrient depletion - Strain antagonism - Increased acidity - Positive redox potential (presence of oxygen) - Presence of antimicrobial compounds; hydrogen peroxide and bacteriocins - Storage temperature
GI transit	- Gastric acid and juices - Bile salts - Microbial antagonism

CHAPTER III

SELECTION AND CHARACTERIZATION OF *BIFIDOBACTERIUM* SPP. AND *LACTOBACILLUS* SPP. THROUGH SIMULATED GASTROINTESTINAL CONDITIONS

3.1 Introduction

A trend in the consumption of health-promoting foods has developed in recent years together with an increasing variety of products conferring specific health benefits. In this regard, probiotic-containing foods are highlighted as attractive products due to superior health promotion effects (Peres et al., 2014; Sousa et al., 2015). The popularity of these products has resulted in research efforts targeted to screen potential probiotic strains. In the search for interesting strains with probiotic potential, it is necessary to provide preliminary *in vitro* screening to ensure safety and the functional aspects. The functional requirements of probiotics include tolerance to acid and bile stresses, adherence to epithelial surfaces, and antagonistic activity towards intestinal or food-borne pathogens (Ramos et al., 2013). According to the FAO/WHO (FAO/WHO, 2006), probiotics are currently defined as "live microorganisms which when administered in adequate amounts confer beneficial effects on the health of the host." However, before probiotics being of benefit to human health, they need to possess the ability to survive in the product in sufficient

numbers throughout the manufacturing processes and storage, including during passage through GI tract, to be effective to the host (Saarela et al., 2010). Consequently, probiotic strains selected for commercial applications must retain the characteristics for which they were originally selected (Sharma et al., 2014). The selection of local potential strains from natural sources may constitute a promising approach to obtain useful and genetically-stable strains for industrially-important products (Solieri et al., 2014). In this context, the genus *Bifidobacterium* and *Lactobacillus* were found to be especially interesting because they occupy a wide range of natural and human environments and have a historic use to contribute positive effects and safety record (Solís et al., 2010). Bifidobacteria and lactobacilli are therefore accepted and widely used as health-promoting or probiotic components in functional food products (Andriantsoanirina et al., 2013).

The most typical food vehicles for the delivery of probiotics are dairy products, particularly fermented milk. It was reported that probiotic bacteria are normally commercialized as lyophilized cultures and are mainly added to food products (Vinderola et al., 2012). The incorporation of the probiotics in food preparation requires initial quality regarding the levels of viable bacteria and storage stability throughout the shelf-life of the products (Saarela et al., 2010). For whole processes, the candidate probiotcs are thus subjected to stressful environmental challenges not only during industrial processes such as freeze-drying, manufacturing, and storage, but also after consumption through GI tract stresses until their adherence to the intestinal epithelium to exert health-promoting effects there (Ventura et al., 2007). Therefore, to possess functional and effective probiotic strains with predictable and measurable health benefits, a rigorous effort for strain selection and

characterization is required.

The aim of the present work was to investigate the probiotic characteristics of *Bifidobacterium* and *Lactobacillus* isolated from faecal samples of healthy Thai infants and from traditional fermented foods, respectively. The candidate strains were initially screened on the basis of acid and simulated gastric tolerance and were further screened for functional properties, such as antimicrobial activity and adhesion ability. The selected potential strains were then assessed to a dynamic *in vitro* model through simulated GI stress conditions. In addition, this study considers to be a pioneer work in the evaluation of the impact of the production process chain on the selected probiotic's survival and resistance to GI stress, and its adhesion ability to Caco-2 cells. This part of the study was to ensure that the strain would still provide probiotic effects after consumption.

3.2 Materials and methods

3.2.1 Isolation of bifidobacteria and lactobacilli

Fifteen faecal samples from volunteers of breast-fed Thai infants were collected and processed anaerobically in a laboratory within 2 h for isolation of *Bifidobacterium* strains. Twenty indigenous and traditional fermented foods from different region in Thailand, such as pickled fish**,** pickled cabbage**,** pickled bamboo shoots and fermented sausages, were used as a source for screening of *Lactobacillus* strains. The sample solutions were homogenized and serially diluted in phosphate buffered saline (PBS; 0.8% NaCl, 0.2% KCl, 0.144% Na_2HPO_4, 0.024% KH_2PO_4, pH 7.2) supplemented with 0.05% L-cysteine hydrochloride (Merck, Germany) (PBSc). The solutions were then placed on DeMan, Rogasa and Sharpe (MRS; Oxoid Ltd.,

UK) agar plates supplemented with 0.05% L-cysteine hydrochloride (MRSc). After incubation under anaerobic conditions at 37°C for 24-48 h, the isolates were initially screened on the basis of catalase activity, Gram staining, and morphology. All catalase-negative, Gram-positive with club- or Y-shaped rods resembling branches were tentatively considered as bifidobacteria while Gram-positive with rod-shaped were considerd as lactobacilli. The isolates were purified and maintained in MRSc broth containing 20% (v/v) sterile glycerol and stored at -80°C. The widely used *Bifidobacterium animalis* subsp. lactis, strain BB-12 (BB-12) (Chr. Hansen, Denmark) was included in experiments for comparison purposes.

3.2.2 Screening of probiotic properties

3.2.2.1 Resistance under conditions simulating human GI tract

The resistance of the examined isolates under conditions simulating GI tract was tested as previously described (Maragkoudakis et al., 2006). The tolerance of isolates was initially screened through low pH and simulated gastric juice. Briefly, bacterial cells from overnight (16-18 h) cultures were harvested (4,000 rpm, 10 min, 4°C) and washed twice with PBSc (pH 7.2), before being re-suspended in PBSc solution and adjusted to a pH solution of 2.0 and 3.0 for bifidobacteria and 2.5 and 3.0 for lactobacilli. For resistance to simulated gastric juice, bacterial cells were harvested and washed twice. The bacterial suspension was then re-suspended in PBSc solution containing 0.3% (w/v) pepsin (Sigma-Aldrich, USA), adjusted to pH as described above. Resistance was assessed in terms of viable colony counts on MRSc agar after incubation of bacterial suspensions at 37°C for 0 and 3 h, reflecting the time spent by food in the stomach.

For resistance to small intestine conditions, bacterial cells as prepared above were re-suspended in PBSc solution containing 0.1% (w/v) pancreatin (Sigma-Aldrich, USA) and pH 8. The ability of the isolates to grow in the presence of bile was determined by adding cell suspensions to MRSc broth supplemented with 0.3, 0.5 and 1% (w/v) bile salt (Oxoid Ltd., UK) and pH 8. The viable colony counts were determined after incubation at 37°C for 0 and 4 h, reflecting the time spent by food in the small intestine.

3.2.2.2 Identification of isolates by carbohydrate utilization and 16S rDNA gene sequencing

Phenotypic and genotypic identifications were performed by studying carbohydrate fermentation profiles and partial sequencing of the 16S rDNA gene, respectively. Carbohydrate fermentation patterns of strains were obtained by API 50 CH strips (Bio-Mérieux, France) according to the manufacturer's instructions. An rDNA gene of isolates was amplified using primers Bif164-F in combination with Bif662-R primer specific to 16S rDNA of the *Bifidobacterium* genus (Kok et al., 1996). Members of the *Lactobacillus* genus were investigated for their 16S rDNA by using Bact-0011f in combination with Lab-0677r primers (Heilig et al., 2002). Amplified PCR products were later sequenced (Macrogen, Korea) and the compared sequences showed similarity with those found in the National Center for Biotechnology Information (NCBI) database using BLAST algorithm.

3.2.2.3 *In vitro* adherence assay

An adherence of the selected strains was examined *in vitro* using Caco-2, a colonic adenocarcinoma cell line that expresses the morphological

and physiological characteristics of normal mature human enterocytes. An adhesion assay was conducted as previously indicated by Pennacchia et al. (2006). Caco-2 cells were routinely grown in Dulbecco's modified Eagle's minimal essential medium (DMEM; Gibco, USA) supplemented with 10% (v/v) heat inactivated fetal bovine serum, 1% (v/v) L-glutamine, 1% (v/v) non-essential amino acid solution, and 1% (v/v) penicillin/streptomycin solutions (Gibco, USA) at 37°C in 5% CO_2 and 95% air atmosphere. Before the adhesion assay, overnight cultures of bacterial strains were harvested by centrifugation (4,000 rpm, 10 min, 4°C). An aliquot of culture suspensions was serially diluted 10-fold in PBSc to determine the viable population by plate counting on MRSc agar after 48 h of incubation at 37°C. Another aliquot was re-suspended in non-supplemented DMEM (pH 7.0). This bacterial suspension was used to inoculate the six-well tissue culture plates with a concentration of about 10^8 CFU/mL.

The monolayer Caco-2 cells in the six-well tissue culture plates were washed twice with PBS and 2 ml of non-supplemented DMEM was added to each well. The plate was incubated at 37°C for 1 h. After incubation, non-supplemented DMEM was removed from each well and replaced by 1 ml of the bacterial suspension, prepared as described above. After incubation at 37°C for 90 min, the wells were softly washed 3 times with PBS to remove non-adherent bacteria. The washed monolayer was treated with 1 ml of 0.05% Triton X-100 water solution for 10 min to lyse the Caco-2 cells. The number of viable adhering bacteria was determined by plating serial 10-fold dilutions of the mixture containing lysed Caco-2 cells and bacterial cells on MRSc agar after 24-48 h of incubation at 37°C. The adhesion ability of the s trains on Caco-2 cells was calculated as a percentage of the

viable bacteria according to their initial population.

3.2.2.4 Antibiotic susceptibility test

Antibiotic susceptibility patterns of the selected strains were investigated by the disk diffusion method. The tested antibiotic discs (Oxoid, England) included streptomycin (10μg), gentamicin (10μg), tetracycline (30μg), penicillin G (10μg), aztreonam (30μg), vancomycin (30μg), erythromycin (15μg), chloramphinicol (30μg), kanamycin (30μg), ampicilin (10μg), lincomycin (15μg), norfloxacin (10μg), and ofloxacin (5μg). Concentration of antibiotics were selected as recommended by the Clinical and Laboratory Standards Institute (CLSI, 2014). Strains were grown overnight in MRSc broth at 37°C under anaerobic conditions to obtain a density of 10^7 CFU/mL. The culture suspension was swabbed on MRSc agar. Antibiotic discs were placed aseptically on the inoculated plates and agar plates were incubated anaerobically for 24 h at 37°C. The diameters of the inhibition zones around the discs were measured (average of three readings) and the results were interpreted according to CLSI as sensitive (S), intermediate (I), and resistant (R).

3.2.2.5 Antimicrobial Activity

The ability of the candidate strains to inhibit the growth of pathogenic microorganisms was determined using the agar-well diffusion assay (Nami et al., 2014). The indicator organisms used in the current study included *Escherichai coli* TISTR 780, *Staphylococcus aureus* TISTR 1466, *Pseudomonas aeruginosa* TISTR 781, *Bacillus cereus* TISTR 687, *Samonella typhimurium* TISTR 292, *Vibrio cholera* O139, and *Candida albicans* TISTR 718, and these were obtained

from the culture collection of the Laboratory of Microbiology, Institute of Science, Suranaree University of Technology. An overnight culture of the indicator strains was applied to inoculate in Brain-Heart Infusion agar (BHI; Conda-Pronadisa, Spain) at 37°C. Fresh overnight bifidobacteria and lactobacilli cultures were harvested by centrifugation (4,000 rpm, 10 min, 4°C). The supernatants were neutralized to pH 6.5 and the other left unadjusted followed by filter-sterilization through 0.22 μm membrane filter. Cell-free extracts of each sample (100 μL) were pipetted into drilled holes (7mm) of the agar. The plates were then incubated at 37°C and were examined after overnight incubation. Antimicrobial activity was recorded as growth free inhibition zones (mm) around the well.

3.2.2.6 Storage stability of probiotics in commercial products during storage

All candidate bifidobacteria were propagated in MRSc broth overnight at 37°C followed by sub-culturing and incubating for a further 18 h. All cultures were harvested by centrifugation and the pellets were then washed twice in PBSc solution, pH 7.4. A 1% inoculum of each bifidobacterial culture was aseptically distributed into 100 mL portions of four commercial dairy and non-dairy products (pasteurized milk, drinking yogurt, soymilk, and orange juice) to obtain a final concentration of 10^7-10^8 CFU/mL. Cell counts and pH measurements were performed immediately after the addition and every three days until 15 days of storage at a refrigerated temperature (≈ 4°C).

3.2.2.7 GI transit tolerance

The viability of selected strains was assessed through dynamic *in vitro* model intended to GI stress conditions. To mimic *in vivo* human GI transit, an *in vitro* model was conducted as previously described by Peres et al. (2014) and Sousa et al. (2015), with slight modifications. After two successive transfers at 37°C for 16 h were transferred to a 34 ml of sterile electrolyte solution (SES; 0.22 g L^{-1} $CaCl_2$, 6.2 g L^{-1} NaCl, 2.2 g L^{-1} KCl, 1.2 g L^{-1} $NaHCO_3$, w/v) adjusted to pH 6.2. To simulate *in vivo* saliva conditions, 5 mL of a sterile electrolyte solution containing lysozyme (final concentration of 0.01% w/v) was added to 35 mL of cell suspension and incubated at 37°C, 200 rpm for 2 min. Then, 3 mL of the electrolyte solution (pH 5.0) with 0.3% (w/v) pepsin was incorporated into the cell suspension to simulate the oesophagus-stomach environment. The pH curve in the stomach was reproduced by adding 1 N HCl to the cell suspension to pH 6.0, 5.0, 4.0 every 10 min and to pH 3.0, 2.0 every 30 min, at 37°C, 50 rpm, respectively. After 90 min of incubation, the samples were then adjusted to pH 5.0 using 1 M $NaHCO_3$ and mixed with 4 mL of a sterile electrolyte solution (5 g/L NaCl, 0.6 g/L KCl and 0.3 g/L $CaCl_2$, w/v), containing 0.3% (w/v) bile salts and 0.1% (w/v) pancreatin (pH 8) and incubated for 30 min (37°C and 50 rpm) to simulate the intestinal environment at the duodenum step. Finally, the ileum step was brought about by increasing the pH to 6.5 and incubation for 90 min at 37°C and 50 rpm. Changes in total viable counts by the end of each stage of digestion were also monitored.

3.2.3 Preservation of bifidobacteria by freeze-drying in different cryoprotectants

Fresh overnight culture of a selected probiotic strain, *B. animalis* BF052, was grown in MRSc broth at 37°C. A 1% inoculum was then subsequently transferred to fresh MRSc broth. At the early stationary phase of growth (18 h), cells were harvested by centrifugation and washed twice with PBSc solution, pH 7.4. The pellet was re-suspended in 10% (w/v) lactose, 10% (w/v) sucrose, 10% (w/v) skim milk, 10% (w/v) germinated brown rice (GBR), 10% (w/v) black sesame (BS), and commercial soymilk. Sterile de-ionized water was used as a control. Aliquots (1 ml) of each cell suspension in different cryoprotectants were transferred into sterilized vials and frozen at -80°C for 4 h. Then, the samples were immediately freeze-dried for 18 h in a freeze-dryer (Alpha 1-2, Christ, Germany).

After freeze-drying, the freeze-dried powders were re-hydrated with MRSc broth (1 ml) and the cell suspensions were allowed to stand for 10 min at room temperature and subsequently plated on MRSc agar. The number of viable cells before and after freeze-drying was determined at 37°C after incubation for 48 h. To select the most effective cryoprotectant, freeze-dried samples were kept at room (≈ 25°C) and refrigerated temperatures (≈ 4°C). After storage for 1, 3, and 6 months, the viability of the freeze-dried cells was then enumerated by plating on MRSc agar after 48 h of incubation at 37°C.

3.2.4 Effect of food processing chain on probiotic properties during GI transit

The study investigated the effects of the production process chain, freeze-drying, storage of freeze-dried powders, and incorporation of cells in food matrix on the stability of the probiotic properties of *B. animalis* BF052. The strains of *B. animalis* BF052 were passed through a process of freeze-drying by using 10% skim milk as a cryoprotective agent and the freeze-dried powders were then stored for 1 month following incorporation into milk and kept at refrigerated temperatures for 2 weeks. The strain was then sequentially exposed to GI transit followed by the adherence assay as previously described in **3.2.2.3**. Table 3.1 summarizes the stages occured during GI transit.

Table 3.1 *In vitro* model of GI conditions.

Compartment	Conditions	Agitation (rpm)	pH	Time (min)
Mouth	0.01% (w/v) lysozyme	200	6.2	2
Oesophagus-stomach	0.3% (w/v) pepsin,	130	ΔpH 2- 6 6 5 4 3 2	90 10 10 10 30 30
Duodenum	0.3% (w/v) bile salts and 0.1% (w/v) pancreatin,	50	5	30
Ileum	-	50	6.5	90
Intestinal mucosa	Caco-2 cells	-	7	90

3.2.5 Statistical analysis

Data were analyzed using SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). Statistical differences in multiple groups were determined by one-way ANOVA followed by multiple mean comparisons with Duncan's test. All numerical data were displayed as mean ± standard deviation and $p \leq 0.05$ was considered statistically significant.

3.3 Results and discussion

3.3.1 Screening of probiotic properties of bifidobacteria

3.3.1.1 Isolation of bifidobacteria

The collected faecal samples from breast-fed Thai infants were kept and analyzed for the isolation of bifidobacteria. After incubation, most of the colonies that appeared on MRSc screening plates were cream to white in color, translucent to opaque, and round with an entire margin and convex elevation (Figure 3.1). A total of 325 isolates were catalase-negative, Gram-staining-positive and irregularly shaped rods, Y-shaped, or club-shaped, were tentatively considered as bifidobacteria (Figure 3.2)

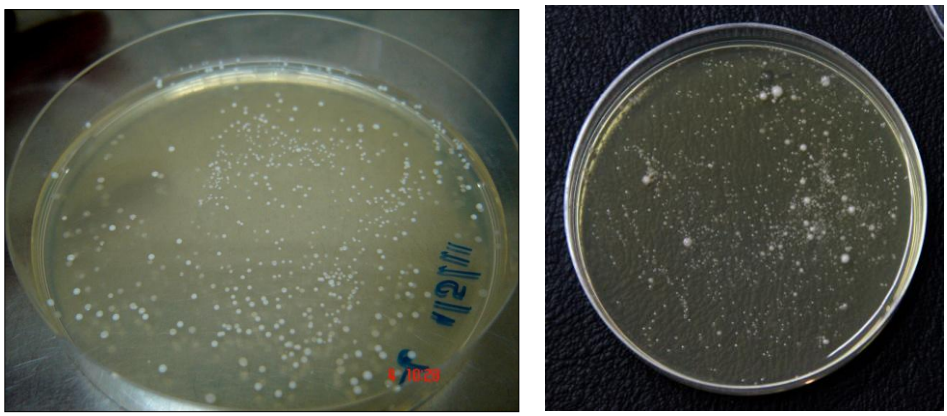


Figure 3.1 Colony morphology on MRSc screening plates.

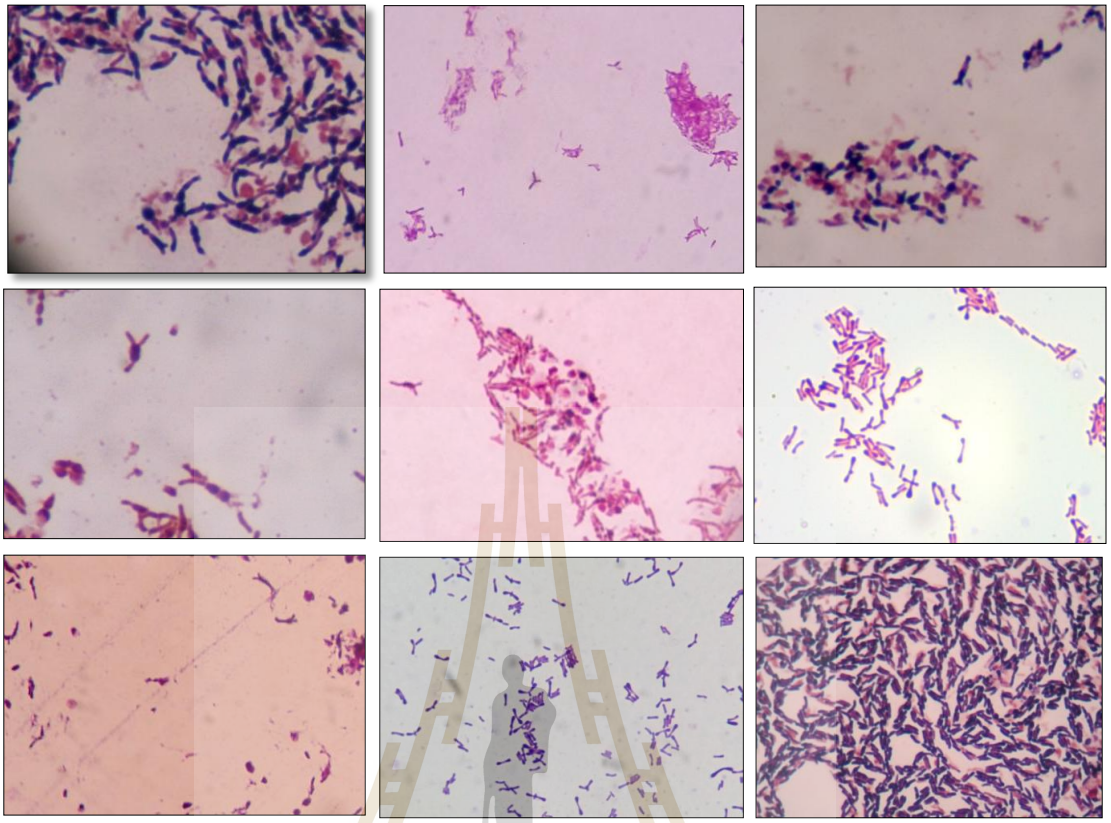


Figure 3.2 Cell morphology of isolated bifidobacteria under microscope (100X).

3.3.1.2 Resistance of bifidobacteria under conditions simulating GI tract

An essential step towards the selection of potential probiotic candidates is to examine their resistance under GI stress environments (Garcia-Ruiz et al., 2014; Peres et al., 2014). In the present study, tolerance to low pH and simulated gastric conditions were chosen as selection criteria to reduce the number of non-tolerant isolates. The acid-tolerant levels of the isolated strains are shown in Table 3.2. Out of 325 strains representing bifidobacterial morphology, only 4 strains (BF014, BF049, BF052 and BH053, including reference strain BB-12) showed a decrease in viable counts lower than 1 log cycle even after 3 h of exposure at pH 3.

No significant differences ($p>0.05$) in viable cells were observed in BF052 and BB-12 after exposure in solutions with or without pepsin at pH 3, compared with initial counts. Although BF014, BF052 and BH053 did not survive after exposure to pH 2 for 3 h, all of the three strains consistently tolerated the pepsin solutions at pH 2 after 3 h of incubation. These results indicated that the bifidobacterial isolates (except strain BF049) were able to tolerate simulated gastric juice, which combined the effect of a pepsin-pH solution which was greater than low pH conditions. This result is relevant to the work of Mättö et al. (2006) that the addition of inhibitors of pepsin and proton translocating enzyme significantly decreased the survival rate of *B. animalis* subsp. *lactis* at pH 2. Therefore, it was likely that pepsin was able to protect the cells during exposure to low pH by maintenance of the pH homeostatis and support of the role of H^+-ATPase. However, the loss of viability in BF049 after exposure to simulated gastric juice may indicate that the resistance to enzymatic barriers was strain-specific. In this study, even strains were not able to survive at pH 2 *in vitro*, they may exhibit the substantial viability when they are consumed as starters or adjuncts in other carrier matrix. Rubio et al. (2014) suggested that the consumption of probiotics with other food matrices was not likely to expose probiotics alone to the extremes of pH in the stomach.

The strains resistant to stomach conditions were further tested for their ability to tolerate small intestinal conditions. All of the candidate strains were resistant to bile salts and pancreatic solutions at pH 8 by decreasing their viability approximately 1 log unit after 4 h exposure, as shown in Table 3.3. In general, the relevant physiological concentrations of human bile range from 0.3% to 0.5%. However, it was reported that bile salts were critical to bacterial cells since they

disorganized the structure of the cell membrane (Kaewnopparat et al., 2013). However, all tested strains in this study retained their viability with small reductions at high concentrations of bile ranges from 0.3% to 1%.

Table 3.2 Cell viability of probiotic strains after 3 h of exposure to low pH conditions and simulated gastric juice.

Strain	Cell count (log CFU/mL ± SD)[φ]				
	Initial	Low pH condition		Resistance to gastric juice with 0.3% (w/v) pepsin	
		pH 2	pH 3	pH 2	pH 3
BB-12	6.71 ± 0.02[a]	-[γ]	6.61 ± 0.05[a]	6.21 ± 0.04[b]	6.65 ± 0.01[a]
BF014	7.14 ± 0.11[a]	-	6.86 ± 0.25[ab]	6.46 ± 0.05[b]	7.08 ± 0.12[a]
BF049	7.13 ± 0.03[a]	-	6.93 ± 0.02[b]	-	1.61 ± 0.02[c]
BF052	7.31 ± 0.06[a]	-	7.25 ± 0.07[a]	6.99 ± 0.02[b]	7.26 ± 0.05[a]
BH053	7.79 ± 0.06[a]	-	7.34 ± 0.18[b]	7.19 ± 0.03[b]	7.65 ± 0.03[a]

[φ] Each value represents the mean value (log CFU/mL) ± standard deviation (SD) from three trials. The equal superscript lowercase letters in the same row indicate no significant differences ($p>0.05$).

[γ] No growth

In this study, three isolates, BF014, BF052, and BH053, showed satisfactory probiotic properties for preliminary screening under conditions simulating GI tract, suggesting that they may survive through the human GI transit. All of these three strains were therefore selected for the study of other probiotic properties.

Table 3.3 Cell viability of probiotic strains after 4 h of exposure to bile salt and pancreatin.

Strain	Cell count (log CFU/mL ± SD)[φ]				
	Initial	Bile salt (pH 8)			Pancreatin (pH 8)
		0.3%	0.5%	1.0%	
BB-12	7.42 ± 0.14[a]	6.45± 0.08[b]	6.43 ± 0.04[b]	6.41 ± 0.09[b]	6.42 ± 0.27[b]
BF014	7.28 ± 0.12[a]	6.51 ± 0.26[b]	6.38 ± 0.03[b]	6.43 ± 0.02[b]	6.15 ± 0.14[b]
BF049	7.27 ± 0.08[a]	6.58 ± 0.03[b]	6.58 ± 0.07[b]	6.32 ± 0.09[c]	5.10 ± 0.11[d]
BF052	7.44± 0.06[a]	6.80 ± 0.02[b]	6.77 ± 0.01[b]	6.73 ± 0.03[b]	6.32 ± 0.15[c]
BH053	7.43 ± 0.10[a]	6.25 ± 0.18[b]	6.23 ± 0.12[b]	6.23 ± 0.15[b]	6.31 ± 0.15[b]

[φ] Each value represents the mean value (log CFU/mL) ± standard deviation (SD) from three trials. The equal superscript lowercase letters in the same row indicate no significant differences ($p>0.05$)

3.3.1.3 Identification of isolates by carbohydrate utilization and 16S rDNA gene sequencing

An analysis of carbohydrate fermentation by the selected strains was done using the API 50 CHL system kit. All the tested strains, including BB-12, fermented the following carbohydrates; D-ribose, D-glucose, amygdalin, esculin, salicin, D-maltose, D-melibiose, D-sucrose, D-raffinose, and gentiobiose. On the basis of the 16S rDNA gene analysis, approximately 520 bp of PCR product was amplified and sequenced. All of the three isolates were identified as *Bifidobacterium animalis* (100% similarities for BF014 and BH053 and 99% for BF052) as shown in Table 3.4. The similarity pattern of the phenotypic results confirmed the identity obtained by 16S rRNA gene sequencing, indicating that all strains belonged to *B. animalis*.

Table 3.4 The 16S rRNA gene sequences of selected bifidobacteria.

Isolate	Nucleotide sequences	% Identity
BF014 (461 bp)	GTACCCGGCGCAGATCCACCGTTAGGCGATGGACTTTCACACCGG ACGCGACGAACCGCCTACGAGCCCTTTACGCCCAATAAATCCGGA TAACGCTCGCACCCTACGTATTACCGCGGCTGCTGGCACGTAGTT AGCCGGTGCTTATTCGAACAATCCACTCAACACGGCCGAAACCGT GCCTTGCCCTTGAACAAAAGCGGTTTACAACCCGAAGGCCTCCAT CCCGCACGCGGCGTCGCTGCATCAGGCTTGCGCCCATTGTGCAAT ATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGGCCGTATCTCAGTC CCAATGTGGCCGGTCACCCTCTCAGGCCGGCTACCCGTCAACGCC TTGGTGGGCCATCACCCCGCCAACAAGCTGATAGGACGCGACCCC ATCCCATGCCGCAAAAGCATTTCCCACCCCACCATGCGATGGAGC GGAGCATCCG	100% *Bifidobacterium animalis* subsp. lactis BLC1, complete genome: (CP003039.2)
BF052 (461 bp)	GTACCCGGCGCAGATCCACCGTTAGGCGATGGACTTTCACACCGG ACGCGACGAACCGCCTACGAGCCCTTTACGCCCAATAAATCCGGA TAACGCTCGCACCCTACGTATTACCGCGGCTGCTGGCACGTAGTT AGCCGGTGCTTATTCGAACAATCCACTCAACACGGCCGAAACCGT GCCTTGCCCTTGAACAAAAGCGGTTTACAACCCGAAGGCCTCCAT CCCGCACGCGGCGTCGCTGCATCAGGCTTGCGCCCATTGTGCAAT ATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGGCCGTATCTCAGTC CCAATGTGGCCGGTCACCCTCTCAGGCCGGCTACCCGTCAACGCC TTGGTGGGCCATCACCCCGCCAACAAGCTGATAGGACGCGACCCC ATCCCATGCCGCAAAAGCATTTCCCACCCCACCATGCGATGGAGC GGAGCAACCG	99% *Bifidobacterium animalis* subsp. lactis BLC1, complete genome: (CP003039.2)
BH053 (461 bp)	GTACCCGGCGCAGATCCACCGTTAGGCGATGGACTTTCACACCGG ACGCGACGAACCGCCTACGAGCCCTTTACGCCCAATAAATCCGGA TAACGCTCGCACCCTACGTATTACCGCGGCTGCTGGCACGTAGTT AGCCGGTGCTTATTCGAACAATCCACTCAACACGGCCGAAACCGT GCCTTGCCCTTGAACAAAAGCGGTTTACAACCCGAAGGCCTCCAT CCCGCACGCGGCGTCGCTGCATCAGGCTTGCGCCCATTGTGCAAT ATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGGCCGTATCTCAGTC CCAATGTGGCCGGTCACCCTCTCAGGCCGGCTACCCGTCAACGCC TTGGTGGGCCATCACCCCGCCAACAAGCTGATAGGACGCGACCCC ATCCCATGCCGCAAAAGCATTTCCCACCCCACCATGCGATGGAGC GGAGCATCCG	100% *Bifidobacterium animalis* subsp. lactis BLC1, complete genome: (CP003039.2)

3.3.1.4 Caco-2 cell adhesion

Adhesion of probiotic strains to human intestinal mucosa is regarded as a prerequisite characteristic for potential probiotic microorganisms. The adhesion ability to Caco-2 cells was evaluated and the result is presented in Table 3.5. The BF052 strain had a significantly higher adherence (3.38% ± 0.15) to Caco-2 cells comparable with the reference strain BB-12 (2.96% ± 0.12), whereas BF014 and BH053 expressed lower levels of adhesive abilities than those of BF052 and BB-12 strains. Sánchez et al. (2010) revealed that adhesion values to the intestinal cell line

HT29-MTX by *B. animalis* subsp. lactis IPLA4549 (2.96%±1.74) was slightly lower than BB-12 (3.08%±1.37). In addition, Laparra and Sanz (2009) also reported that BB-12 showed the highest adherence capability to Caco-2 cell and to human mucus (mucin type II) compared with other probiotic strains including *Lactobacillus rhamnosus* GG, *B. animalis* IATA-A2 and *B. bifidum* IATA-ES2. In this study, BF052 showed the highest percentage of adhesion than those candidate strains including the reference strain. As previously reported, the adhesion capability was not associated with species but as a characteristic of strain (López et al., 2012).

The adhesion of the microorganisms to the intestinal mucosa is an important feature involved in colonization and is related to the ability of the strains to interact with the host (González-Rodríguez et al., 2013). Probiotic bifidobacteria have several mechanisms that enable them to adhere to the intestinal epithelial cells. Their possible mechanisms may confer competition for substrates, direct antagonism by inhibitory substances, competitive exclusion of pathogenic bacteria, and potentially host-mediated effects, such as enhancing the function of the intestinal epithelial barrier by stimulation of the various signaling pathways and modulating immune responses (Lebeer et al., 2010; O'Toole and Cooney, 2008; Wang et al., 2010). As a result, high adhesive ability of bacteria to the cell lines may indicate that strains may contribute their beneficial effects to the host. However, *in vivo* investigations are still necessary to confirm their functionality in *in vivo* situations.

Table 3.5 Adhesion ability of the isolates to Caco-2 cells.

Strain	% adhesion (mean±SD)[φ]
BB-12	2.96 ± 0.12^{a}
BF014	2.57 ± 0.38^{b}
BF052	3.38 ± 0.15^{c}
BH053	2.72 ± 0.37^{ab}

[φ] The equal superscript lowercase letters in the column indicate no significant differences between strains ($p>0.05$).

3.3.1.5 Antibiotic susceptibility assay

An important requirement for probiotic strains is that the isolated probiotics must be safe for human consumption. In this regard, antibiotic susceptibility profiles should be revealed and taken into account for safety (Arboleya et al., 2011). Table 3.6 lists the antibiotic susceptibility patterns of the candidate isolates and all candidate strains displayed similar phenotypic resistances comparable with the reference strains, BB-12. All tested strains were interpreted to be resistant towards aminoglycoside group (streptomycin, gentamycin, kanamycin), fluoroquinolone antibiotics (norfloxacin and ofloxacin), and β-lactam antibiotic (aztreonam, which is Gram-negative spectrum). In contrast, all strains were sensitive to antibiotics belonging to a broad range of antibiotics related to different modes of action, such as β-lactam antibiotics (penicillin and ampicilin), broad-spectrum antibiotics (tetracycline and chloramphenicol), macrolide antibiotic (erythromycin), glycopeptide antibiotic (vancomycin), and lincosamide antibiotic (lincomycin). These antibiotic results indicated related patterns to previous reports (Ammor et al., 2007;

D'Aimmo et al., 2007; Sharma et al., 2014). Figure 3.3 shows an example of antibiotic susceptibility pattern of strain BF052.

Table 3.6 Antibiotic susceptibility profiles.

Type of antibiotics	**Antibiotic susceptibility profiles**			
	BB-12	**BF014**	**BF052**	**BH053**
Streptomycin (10μg)	R	R	R	R
Gentamicin (10μg)	R	R	R	R
Tetracycline (30μg)	S	S	S	S
Penicillin G (10μg)	S	S	S	S
Aztreonam (30μg)	R	R	R	R
Vancomycin (30μg)	S	S	S	S
Erythromycin (15μg)	S	S	S	S
Chloramphenicol (30μg)	S	S	S	S
Kanamycin (30μg)	R	R	R	R
Ampicilin (10μg)	S	S	S	S
Lincomycin (15μg)	S	S	S	S
Norfloxacin (10μg)	R	R	R	R
Ofloxacin (5μg)	R	R	R	R

S: Sensitive and R: Resistant

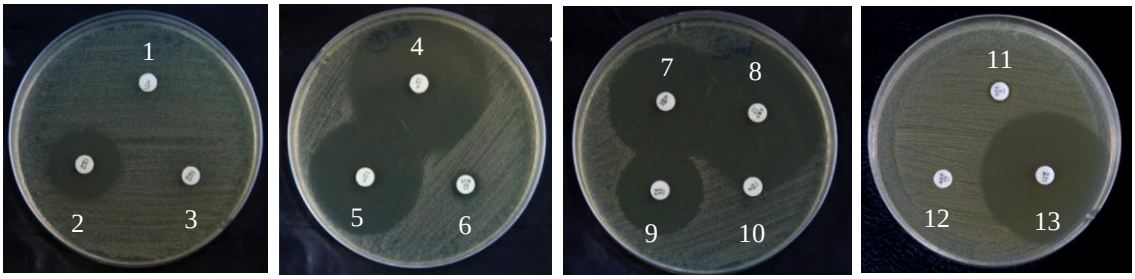


Figure 3.3 Antibiotic susceptibility pattern of strain BF052. No.1-13 represent streptomycin, tetracycline, gentamicin, penicillin G, erythromycin, aztreonam, chloramphenicol, ampicilin, vancomycin, kanamycin, ofloxacin, norfloxacin and lincomycin, respectively.

From a safety point of view, it was proposed that a prospective probiotic should not carry transmissible antibiotic resistant genes, resulting in the corresponding genes not being transferred to the others including pathogens and commensal gut microbiota (Bujnakova et al., 2014). Probiotic strains with intrinsic antibiotic resistance may be thus useful for the restoration of the gut microbiota after antibiotic treatment (Sharma et al., 2014). Moreover, to the best of our knowledge, this is the first report in which all tested strains conferred resistance to norfloxacin and ofloxacin. Therefore, it is beneficial for patients suffering from urinary tract infection to restore the *Bifidobacterium* population after treatments involving norfloxacin and ofloxacin.

3.3.1.6 Antimicrobial Activity

For the antimicrobial assay, there was no observation of inhibition for any of the supernatants in which the pH was neutralized (results not

shown). However, the non-neutralized culture supernatants of BF052 and BH053 strains showed inhibitory activities against *S. typhimurium* and *V. cholerae* as shown in Table 3.7. Figure 3.4 shows antagonistic activity of strains BF052 and BH053 against *V. cholerae*. These results indicated that the most likely explanation was that the inhibition was due to organic acid production by the strains. Our results were in an agreement with previous works. Strompfová and Lauková (2013) demonstrated that inhibition effects were not explained by bacteriocin action and were most probably due to the production of organic acids along with pH lowering effects during the growth in *Bifidobacterium*. Arboleya et al. (2011) also reported that non-neutralized supernatants of breast-milk isolates (*B. longum* and *B. breve*) were able to inhibit *Salmonella enterica* and *Shigella sonnei*. Ibrahim and Bezkorovainy (1993) demonstrated that no antibacterial substances were detected in the fermentation broth of tested bifidobacteria. Only acetic and lactic acids were produced and could inhibit the pathogenic strain of *E. coli*. In addition, Fukuda et al. (2011) proposed that the production of acetate by *B. longum* subsp. longum JCM 1217, *B. longum* subsp. infantis 157F, and *B. longum* subsp. longum NCC 2705 was able to protect mice against death induced by enterohaemorrhagic *Escherichia coli* O157:H7. However, Liu et al. (2016) recently found a novel broad-spectrum bacteriocin called bifidocin A that is produced by *B. animalis* BB04. Therefore, it is likely that the antimicrobial activity of bifidobacteria may be implemented not only by the production of organic acids but also by the secretion of bacteriocin.

Table 3.7 Inhibitory effects of non-neutralized bifidobacterial supernatants against pathogens.

Strains	Diameter (mm) of inhibition zones						
	E. coli	***S. aureus***	***P. aeruginosa***	***V. cholerae***	***B. cereus***	***S. typhimurium***	***C. albicans***
BB-12	-[a]	-	-	-	-	-	-
BF014	-	-	-	-	-	10	-
BF052	-	-	-	8	-	10	-
BH053	-	-	-	9	-	11	-

[a] No antagonistic activity was observed.

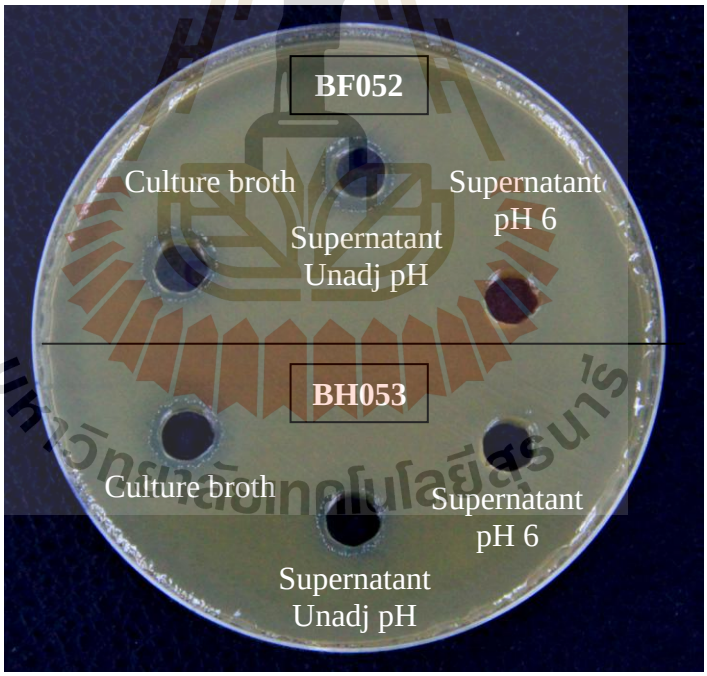


Figure 3.4 Inhibitory effects of bifidobacteria against *V. cholerae*.

3.3.1.7 Storage stability of bifidobacteria in commercial products

Currently, many criteria have been suggested for the selection of probiotics. Besides the challenge to overcome GI stresses, the ability of probiotics to survive in the products during storage is also important. It was recommended that the level of probiotics in food products needed to be high, suggesting the minimum counts of live cells should be at least 10^6-10^7 CFU/mL before consumption (Chaikham, 2015; Sousa et al., 2015). This requirement has a significant impact on the selection of potential probiotics with high stability in different food products.

In the present study, strains BB-12, BF014, BF052 and BH053 were incorporated into dairy (pasteurized milk and drinking yogurt) and non-dairy products (soymilk and orange juice) at refrigerated temperatures for 15 days. Figure 3.5 displays viable cells in refrigerated storage over 15 days. No significant differences ($p>0.05$) were observed in all the candidate strains in cultivable cell numbers during storage in pasteurized milk and soymilk during the 15 days. In drinking yogurt, a significant decrease ($p\leq0.05$) in cell viability was detected only in strain BH053 after storage for nine days. A major significant reduction ($p\leq0.05$) in cell counts ranging from 0.6 to 1.0 log cycles was observed in orange juice in all tested strains. These results are in agreement with those of Saarela et al. (2006) and Vinderola et al. (2012) who reported that the stability of bifidobacterial cells in the low pH of fruit juice was poorer than the fairly neutral pH of milk during refrigerated storage. Nualkaekul et al. (2011) proposed that the presence of protein sources in food matrices may improve the survival of bifidobacteria during refrigerated storage. This was in accordance with the present study's findings that high amounts of proteins in drinking yogurt may have resulted in a higher rate of cell survival than in juices,

although pH values of both products were slightly different. It was proposed that when probiotic cells were present in low pH environments, the requirement of energy consumption increased to maintain the intracellular pH, resulting in depression of ATP for crucial cellular functions and thereby causing cell death. In addition, exposure to oxygen under acidic conditions during refrigeration storage was most probably responsible for the reduction in probiotic counts (Pimentel et al., 2015a; Sheehan et al., 2007). Among all the candidates, BF052 showed the highest survival rate during storage in all products, while the reduction rates of BH053 in terms of viable counts were significantly higher than those of other strains.

Additionally, changes of pH values of BH053 slightly declined compared with those of other strains, especially in soymilk, whereas BF052 remained constant during the incubation period (data not shown). The reduction of the pH value of BF052 in only soymilk (within 0.17 pH-values) but not other products was observed. This result was in line with a previous study which showed the decrease of the pH levels in the soy beverage was faster than in milk (Farnworth et al., 2007; Wang et al., 2004), suggesting a greater rate of organic acid production. It was also observed that soymilk containing oligosaccharides, such as raffinose and stachyose, may support the growth of bifidobacteria causing acid production and subsequent reduction of pH. However, post-acidification during storage is an undesirable property in probiotic-containing products. This process may have adverse effects on the taste or aroma of the product and may cause a loss in the viability of the probiotic strain (Heller, 2001).

The main purpose of the present study was to select the best probiotic bifidobacterial strain for further development as an effective probiotic

starter. It was clearly observed that candidate strains belonging to the same species (*B. animalis*) may present different characteristics even in food matrices. Among all candidates, BF052 was found to exhibit the highest survivability in a wide variety of the products, suggesting that it may have been present in sufficient amounts throughout the entire shelf life of the product. In addition, BF052 possessed considerable probiotic properties including high acid and bile tolerance ability, strong adhesion capability, and good inhibitory activity against pathogens. This strain was thus selected as a promising probiotic strain for futher study.

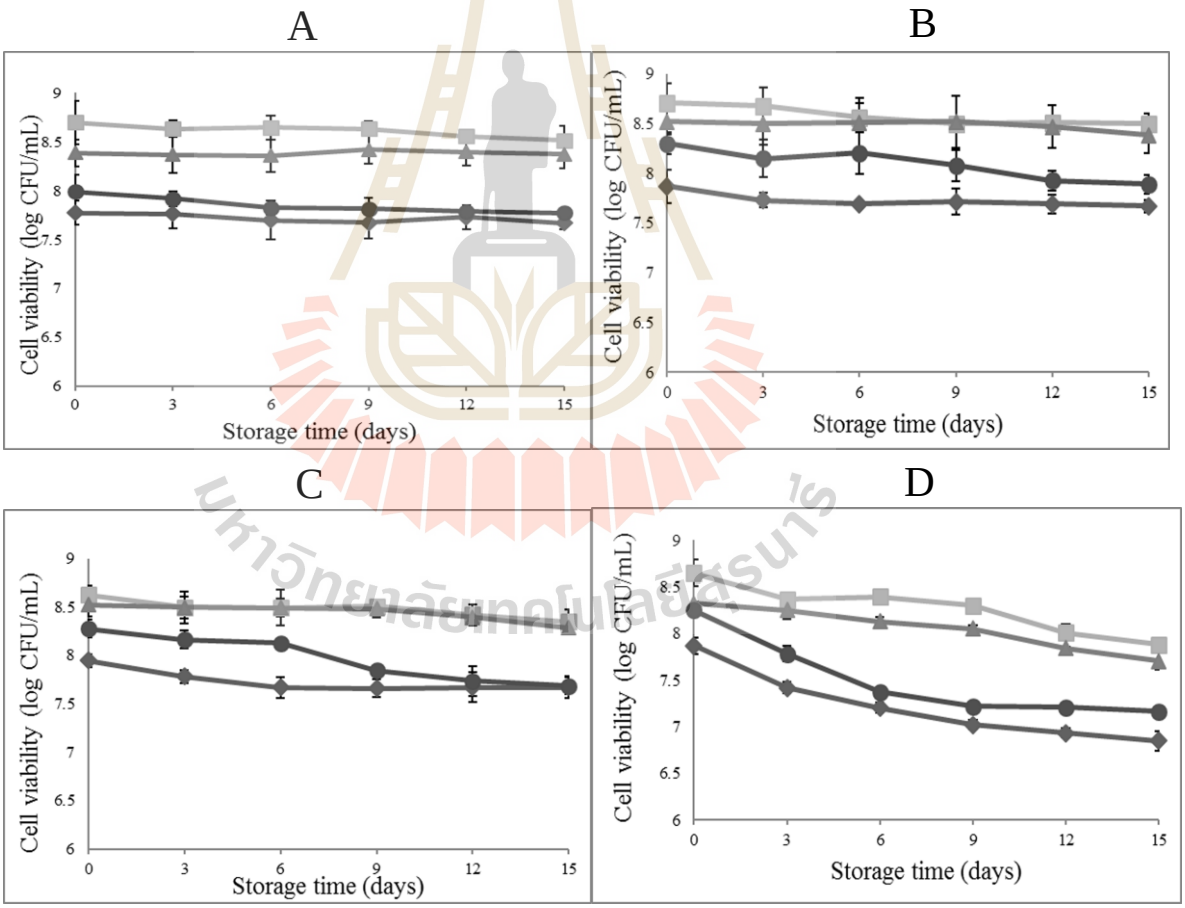
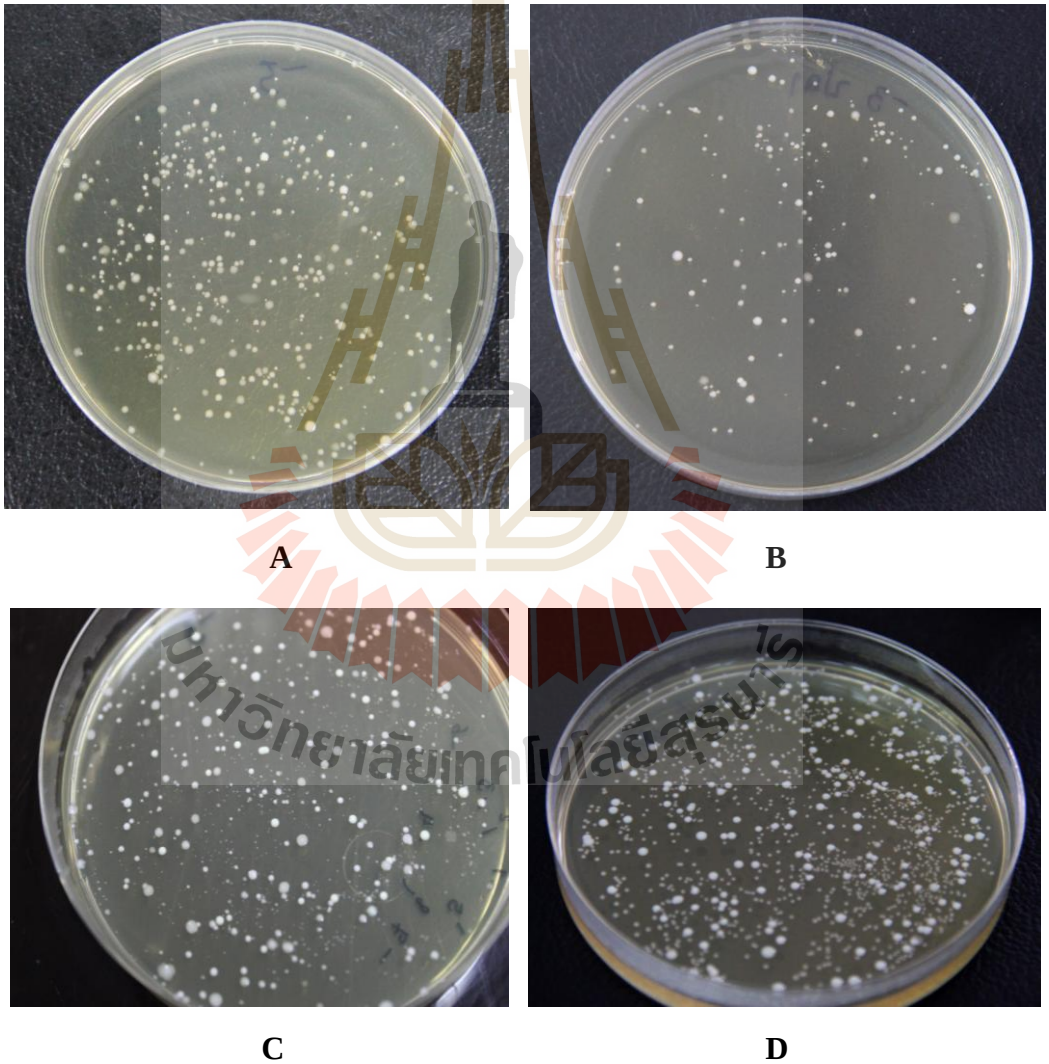


Figure 3.5 Cell viability of bifidobacteria in refrigerated storage over 15 days in (A) pasteurized milk, (B) soymilk, (C) drinking yogurt and (D) orange juice. Symbols: BB-12 (◆), BF014 (■), BF052 (▲), and BF053 (●).

3.3.2 Screening of probiotic properties of lactobacilli

3.3.2.1 Isolation of lactobacilli

More than 300 isolates obtained from traditional fermented foods were tentatively considered as lactobacilli. Figures 3.6 and 3.7 show the morphologies of isolates on MRSc agar plates and under microscope, respectively. All selected isolates were Gram-positive, rod-shaped and catalase-negative.



A **B**

C **D**

Figure 3.6 The appearance of colonies on MRSc plates, isolated from pickle fish and shrimp (A), fermented fish (B), fermented sausages (C) and pickled cabbage (D).

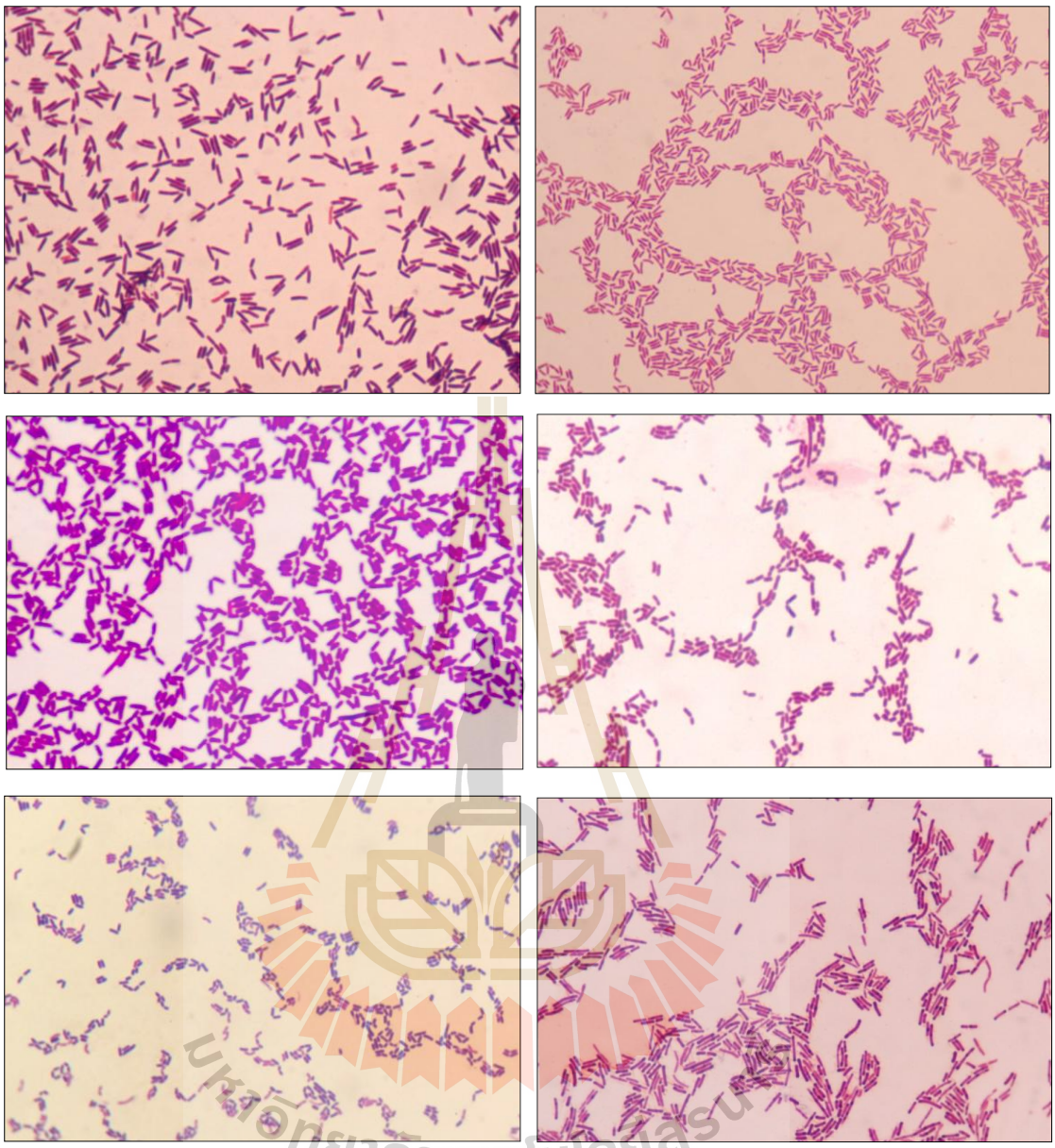


Figure 3.7 Cell morphology of isolated lactobacilli under microscope (100X).

3.3.2.2 Resistance of lactobacilli under conditions simulating GI tract

A preliminary subtractive screening based on the abilities of the isolates to resistant under low pH and simulated gastric conditions was performed to reduce the number of non-tolerant isolates. Among all isolates, LF005, LF022, LF026 and LB013 showed tolerance to pH 2.5 after 3 h of incubation and the residual counts

were greater than 6 log CFU/mL, as shown in Table 3.8. Also, all of these strains were found to survive higher than 7 log CFU/mL under pH 3. However, all of candidates could not survive after exposure at pH 2 for 3 h (data not shown). The results are consistent with those previously recovered from other *Lactobacillus* candidates, in which lactobacilli were viable even after being exposed to pH values of 2.5-4.0, but showed reduced viability at lower pH values (Guo et al., 2012; Tulumoğlu et al., 2014; Wang et al., 2010).

All the candidate strains, except LC016 and LS028, could tolerate greater than 6 log CFU/mL after exposure to simulated gastric juice at pH 2.5. Only LF005, LF022, LF026 and LB013 showed a decrease in viable counts lower than 1 log cycle after 3 h of exposure at pH 3 in gastric condition. Testing the tolerance of lactobacilli isolates under *in vitro* simulated gastric condition could forecast the ability of the strains to survive through the human gastric stress environment (Wang et al., 2010). Based on acid and simulated gastric tolerance results, LF005, LF022, LF026 and LB013 showed great resistant abilities and were therefore selected for bile tolerance test.

Table 3.8 Cell viability of lactobacilli isolates after 3 h of exposure to low pH and simulated gastric conditions.

Strain	Isolation origin	Cell count (log CFU/mL ± SD)[φ]				
		Initial	Low pH condition		Gastric juice with 0.3% (w/v) pepsin	
			pH 2.5	pH 3	pH 2.5	pH 3
LC016	pickled cabbage	8.63 ± 0.06[φ, a]	3.73 ± 0.05[e]	5.16 ± 0.05[d]	5.98 ± 0.13[c]	7.18 ± 0.15[b]
LF005	pickled fish	8.53 ± 0.04[a]	6.29 ± 0.16[c]	8.09 ± 0.07[b]	8.09 ± 0.12[b]	8.30 ± 0.03[b]
LF022	pickled fish	8.90 ± 0.01[a]	6.16 ± 0.22[e]	7.58 ± 0.13[c]	6.71 ± 0.19[d]	8.42 ± 0.14[b]
LF026	pickled fish	8.28 ± 0.05[a]	6.08 ± 0.25[c]	7.72 ± 0.19[b]	8.09 ± 0.06[ab]	8.12 ± 0.20[ab]
LS028	fermented sausages	8.34 ± 0.09[a]	5.05 ± 0.12[e]	6.38 ± 0.20[c]	5.93 ± 0.06[d]	7.18 ± 0.15[b]
LB006	pickled bamboo shoots	8.86 ± 0.10[a]	4.20 ± 0.22[d]	7.38 ± 0.03[b]	6.19 ± 0.15[c]	7.62 ± 0.17[b]
LB013	pickled bamboo shoots	8.37 ± 0.11[a]	7.70 ± 0.02[b]	8.27 ± 0.15[a]	7.79 ± 0.25[b]	8.17 ± 0.05[a]

[φ] Each value represents the mean value (log CFU/mL) ± standard deviation (SD) from three trials. The equal superscript lowercase letters in the same row indicate no significant differences ($p>0.05$).

The bile tolerant levels of the candidate probiotics were shown in Table 3.9. All candidate strains were resistant to bile salts even after 4 h exposure and retained its viability with small reduction in viable counts (<1 log cycle). No significant reduction ($p>0.05$) in cell viability was observed only in LF022 after exposure to bile ranges from 0.3% to 1% for 4 h. In addition, most of the tested lactobacilli were also resistant to pancreatin by decreasing their viability less than 1 log CFU/mL after 3 h of exposure. Therefore, LF005, LF022, LF026 and LB013 could tolerate well to bile salt and pancreatin solutions and were selected for the study of other probiotic properties.

Table 3.9 Cell viability of lactobacilli isolates after 4 h of exposure to bile salt and pancreatin.

Strain	Cell count (log CFU/mL ± SD)[φ]				
	Initial	Bile salt (pH 8)			Pancreatin (pH 8)
		0.3%	0.5%	1.0%	
LF005	7.66 ± 0.08^{φ,b}	7.89± 0.01^{a}	7.82 ± 0.01^{a}	7.62 ± 0.09^{b}	7.46 ± 0.02^{c}
LF022	7.23 ± 0.09^{a}	7.22 ± 0.01^{a}	7.23 ± 0.02^{a}	7.32 ± 0.03^{a}	7.04 ± 0.03^{b}
LF026	7.11 ± 0.05^{a}	6.78 ± 0.06^{b}	6.61 ± 0.06^{b}	6.21 ± 0.01^{b}	7.03 ± 0.03^{a}
LB013	7.89± 0.02^{a}	7.99 ± 0.04^{a}	7.97 ± 0.02^{a}	7.35 ± 0.14^{b}	6.50 ± 0.12^{c}

[φ] Each value represents the mean value (log CFU/mL) ± standard deviation (SD) from three trials. The equal superscript lowercase letters in the same row indicate no significant differences ($p>0.05$).

3.3.2.3 Identification of isolates by carbohydrate utilization and 16S rDNA gene sequencing

For genotypic identification using API 50CHL analysis, LF022 was found to belong to *Lactobacillus plantarum* by its capability to utilize D-ribose, D-galactose, D-glucose, D-fructose, D-mannose, D-manitol, methl-α-D-mannopyranoside, N-acetylglucosamine, amygdalin, arbutin, esculin, salicin, D-cellobiose, D-maltose, D-lactose, D-melibiose, D-sucrose, D-trehalose, D-raffinose, gentiobiose, D-turanose and potassium gluconate. However, LF005, LF026 and LB013 were interpreted to be *Lactobacillus fermentum*. All of these 3 strains were able to ferment D-ribose, D-galactose, D-glucose, D-fructose, D-mannose, D-maltose, D-lactose, D-melibiose, D-sucrose, D-raffinose and potassium gluconate. Identification of isolates by API 50CHL was then confirmed by molecular identification. On the basis of the 16S rDNA gene analysis, the 4 isolates were also identified as *L. plantarum* for LF022, while LF005, LF026, and LB013 were found to belong to the *L. fermentum* (Table 3.10).

Table 3.10 The 16S rDNA gene sequences of selected lactobacilli.

Isolate	Nucleotide sequences	% Identity
LF005 (630 bp)	GATTGATGGTGCTTGCACCTGATTGATTTTGGTCGCCAACGAGTGGCGGACGG GTGAGTAACACGTAGGTAACCTGCCCAGAAGCGGGGGACAACATTTGGAAAC AGATGCTAATACCGCATAACAGCGTTGTTCGCATGAACAACGCTTAAAAGATG GCTTCTCGCTATCACTTCTGGATGGACCTGCGGTGCATTAGCTTGTTGGTGGGG TAACGGCCTACCAAGGCGATGATGCATAGCCAAGTTGAGAGACTGATCGGCC ACAATGGGACTGAGACACGGCCCATACTCCTACGGGAGGCAGCAGTAGGGAA TCTTCCACAATGGGCGCAAGCCTGATGGAGCAACACCGCGTGAGTGAAGAAG GGTTTCGGCTCGTAAAGCTCTGTTGTTAAAGAAGAACACGTATGAGAGTAACT GTTCATACGTTGACGGTATTTAACCAGAAAGTCACGGCTAACTACGTGCCAGC AGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGATTTATTGGGCGTAAAG AGAGTGCAGGCGGTTTTCTAAGTCTGATGTGAAAGCCTTCGGCTTAACCGGAG AAGTGCATCGGAAACTGGATAACTTGAGTGCAGAAGAGGGTAGTGGACCG	99% *Lactobacillus fermentum* strain K80 16S ribosomal RNA gene (KT589106.1)
LF022 (630 bp)	GGTATTGGATTGGGTGCTTGCATCATGATTTACATTTGAGTGAGTGGCGAACT GGTGAGTAACACGTGGGAAACCTGCCCAGAAGCGGGGGATAACACCTGGAAA CAGATGCTAATACCGCATAACAACTTGGACCGCAGGGTCCGAGCTTGAAAGA TGGCTTCGGCTATCACTTTTGGATGGTCCCGCGGCGTATTAGCTAGATGGTGG GGTAACGGCTCACCATGGCAATGATACGTAGCCAACCTGAGAGGGTAATCGG CCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGG AATCTTCCACAATGGACGAAAGTCTGATGGAGCAACGCCCCGTGAGTGAAGA AGGGTTTCGGCTCGTAAAACTCTGTTGTTAAAGAAGAACATATCTGAGAGTAA CTGTTCAGGTATTGACGGTATTTAACCAGAAAGCCACGGCTAACTACGTGCCA GCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAA AGCGAGCGCAGGCGGTTTTTTAAGTCTGATGTGAAAGCCTTCGGCTCAACCGA AGAAGTGCATCGGAAACTGGGAAACTTGAGTGCAGAAGAGGACAGTGGACCG	99% *Lactobacillus plantarum* strain LSE 16S ribosomal RNA gene (KU720558.1)
LF026 (630 bp)	GATTGATGGTGCTTGCACCTGATTGATTTTGGTCGCCAACGAGTGGCGGACGG GTGAGTAACACGTAGGTAACCTGCCCAGAAGCGGGGGACAACATTTGGAAAC AGATGCTAATACCGCATAACAACGTTGTTCGCATGAACAACGCTTAAAAGATG GCTTCTCGCTATCACTTCTGGATGGACCTGCGGTGCATTAGCTTGTTGGTGGGG TAACGGCCTACCAAGGCGATGATGCATAGCCAAGTTGAGAGACTGATCGGCC ACAATGGGACTGAGACACGGCCCATACTCCTACGGGAGGCAGCAGTAGGGAA TCTTCCACAATGGGCGCAAGCCTGATGGAGCAACACCGCGTGAGTGAAGAAG GGTTTCGGCTCGTAAAGCTCTGTTGTTAAAGAAGAACACGTATGAGAGTAACT GTTCATACGTTGACGGTATTTAACCAGAAAGTCACGGCTAACTACGTGCCAGC AGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGATTTATTGGGCGTAAAG AGAGTGCAGGCGGTTTTCTAAGTCTGATGTGAAAGCCTTCGGCTTAACCGGAG AAGTGCATCGGAAACTGGATAACTTGAGTGCAGAAGAGGGTAGTGGACCG	99% *Lactobacillus fermentum* strain RCM_84-1 16S ribosomal RNA gene (KX674007.1)
LB013 (630 bp)	ATTGATGGTGCTTGCACCTGATTGATTTTGGTCGCCAACGAGTGGCGGACGGG TGAGTAACACGTAGGTAACCTGCCCAGAAGCGGGGGACAACATTTGGAAACA GATGCTAATACCGCATAACAACGTTGTTCGCATGAACAACGCTTAAAAGATGG CTTCTCGCTATCACTTCTGGATGGACCTGCGGTGCATTAGCTTGTTGGTGGGGT AACGGCCTACCAAGGCGATGATGCATAGCCAAGTTGAGAGACTGATCGGCCA CAATGGGACTGAGACACGGCCCATACTCCTACGGGAGGCAGCAGTAGGGAAT CTTCCACAATGGGCGCAAGCCTGATGGAGCAACACCGCGTGAGTGAAGAAGG GTTTCGGCTCGTAAAGCTCTGTTGTTAAAGAAGAACACGTATGAGAGTAACTG TTCATACGTTGACGGTATTTAACCAGAAAGTCACGGCTAACTACGTGCCAGCA GCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGATTTATTGGGCGTAAAGA GAGTGCAGGCGGTTTTCTAAGTCTGATGTGAAAGCCTTCGGCTTAACCGGAGA AGTGCATCGGAAACTGGATAACTTGAGTGCAGAAGAGGGTAGTGGACTCG	99% *Lactobacillus fermentum* strain RCM_84-1 16S, ribosomal RNA gene (KX674007.1)

3.3.2.4 Caco-2 cell adhesion

The adhesion rates of the selected lactobacilli on the Caco-2 cells were compared with the reference strain, *L. plantarum* WCFS1, as shown in Table 3.11. The result showed that no significant differences in the adhesive percentages ($p>0.05$) were observed between *L. plantarum* strain WCFS1 and LF022,

although the reference strain showed the highest levels of adherence than those candidate strains. The strains *L. fermentum* LF005 and LF026 showed moderated adhesion ability, while the *L. fermentum* LB013 showed the lowest percentage of adhesion. These results indicated that the adhesion capability was not only associated with the species but also related to the characteristic of strain.

Table 3.11 Adhesion ability of the *Lactobacillus* isolates to Caco-2 cells.

Strain	% adhesion (mean±SD)[φ]
WCFS1	9.25 ± 1.38^{b}
LF005	2.77 ± 0.60^{a}
LF022	8.56 ± 2.17^{b}
LF026	2.45 ± 0.70^{a}
LB013	0.53 ± 0.33^{a}

[φ] The equal superscript lowercase letters in the column indicate no significant differences between strains ($p>0.05$).

3.3.2.5 Antibiotic susceptibility assay

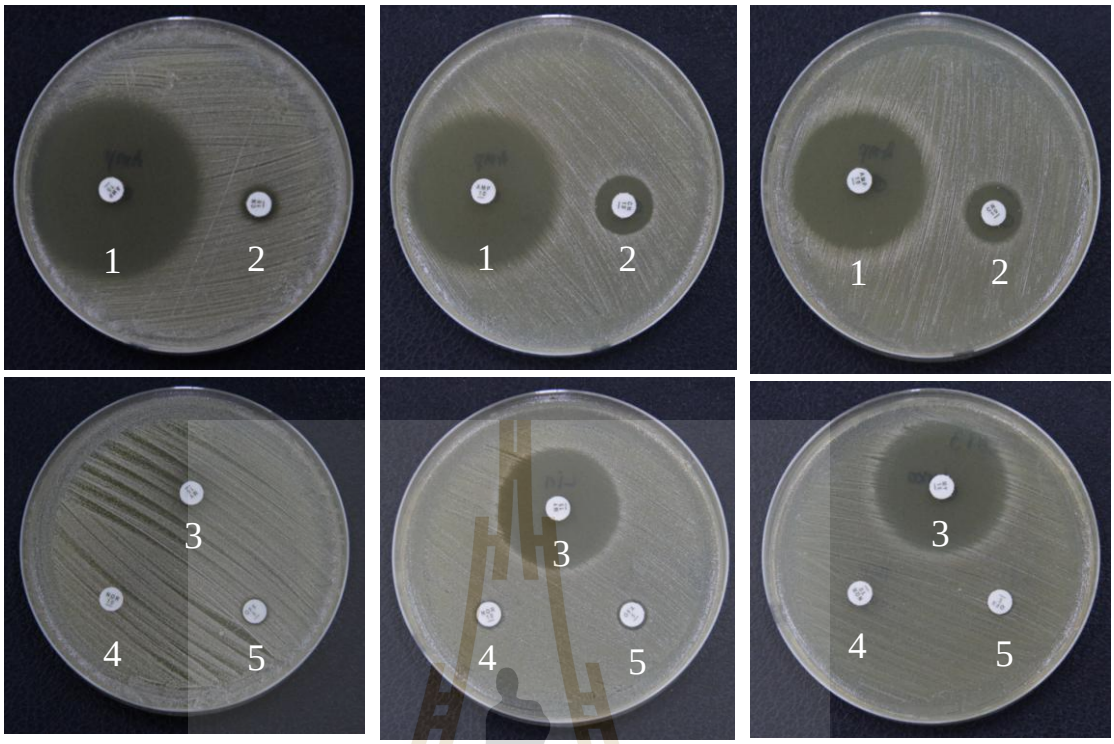
Table 3.12 lists the antibiotic susceptibility patterns of the candidate isolates. All strains displayed several phenotypic resistances comparable with the reference strains, *L. plantarum* WCFS1. All tested strains were interpreted to be resistant towards streptomycin, kanamycin, vancomycin, norfloxacin, ofloxacin and aztreonam. In contrast, all strains were sensitive to antibiotics belonging to penicillin, ampicilin, tetracycline and chloramphenicol. All candidate strains were also classified to be sensitive to erythromycin, which belongs to the macrolide group that inhibiting synthesis of proteins, except reference strain *L. plantarum* WCFS1. It

was reported that the lactobacilli strains were more resistant to antibiotics of the aminoglycoside group (such as kanamycin, gentamycin and streptomycin), however resistance to gentamycin in this study seemed to be strain-specific (Solieri et al., 2014). In addition, only *L. plantarum* LF022 and *L. plantarum* WCFS1 were resistant to lincomycin, indicating that the resistance to lincomycin was also species-dependent (Figure 3.8).

Table 3.12 Antibiotic susceptibility profiles.

Type of antibiotics	**Antibiotic susceptibility profiles**				
	WCFS1	**LF005**	**LF022**	**LF026**	**LB013**
Streptomycin (10μg)	R	R	R	R	R
Kanamycin (30μg)	R	R	R	R	R
Vancomycin (30μg)	R	R	R	R	R
Aztreonam (30μg)	R	R	R	R	R
Norfloxacin (10μg)	R	R	R	R	R
Ofloxacin (5μg)	R	R	R	R	R
Tetracycline (30μg)	S	S	S	S	S
Chloramphenicol (30μg)	S	S	S	S	S
Penicillin G (10μg)	S	S	S	S	S
Ampicilin (10μg)	S	S	S	S	S
Lincomycin (15μg)	R	S	R	S	S
Gentamicin (10μg)	R	I	R	S	S
Erythromycin (15μg)	R	S	S	S	S

S = susceptible, R = Resistant and I = Intermediate resistant



LF022 **LF026** **LB013**

Figure 3.8 Antibiotic susceptibility patterns of the candidate lactobacilli strains. No.1-5 represent ampicilin, gentamicin, lincomycin, norfloxacin and ofloxacin, respectively.

The intrinsic or natural resistance of strains to different classes of antibiotics is probably due to enzymatic inactivation or modification, cell wall structure and membrane impermeability complemented in some cases by their efflux mechanism (Peres et al., 2014; Sharma et al., 2014). This feature might represent a competitive advantage, especially when a probiotic product is administered with antimicrobials for treatment of an infectious disease, thereby reducing likelihood of disbiosis, and rapidly rebalancing normal microbiota.

3.3.2.6 Antimicrobial Activity

Screening of the antagonistic activity of lactobacilli candidates was assessed using agar-well diffusion method (Figure 3.9). The inhibitory ability of the lactobacilli isolates in the form of culture broth and cell free supernatant against pathogenic microorganism is shown in Table 3.13. All of culture broths of candidate strains were able to inhibit the growth of *P. aeruginosa* and *V. cholerae* (Table 3.13). In particular, strains LF005 and LF026 showed strong (≥ 17 mm zone of inhibition) antagonistic activity against *S. aureus* and *P. aeruginosa*. In addition, only culture broths of *L. fermentum* LF005 and *L. plantarum* LF022 exhibited a slight inhibitory activity towards *S. typhimurium*. However, no inhibition was observed for any of the supernatants in which the pH was neutralized (results not shown). Non-neutralized supernatants of all strains, except *L. fermentum* LF026, showed inhibitory activity against only *V. cholerae*, indicated that the antagonistic activity was probably related to organic acid production. In most cases, the inhibition of the pathogens was not maintained when cell free supernatants were tested, indicating that the inhibitory activity might cause from growth compitition. It was reported that certain lactobacilli strains could be used as bioprotective cultures for controlling spoilage and pathogenic microorganisms in fermented products (Rubio et al., 2007). Moreover, lactobacilli also possess roles within such dynamic ecosystems after colonization in GI tract in preventing infection caused by pathogenic organisms which include production of antimicrobial compounds, alteration of intestinal bacterial metabolic activity, alteration of ecology and inhibition of bacterial translocation (Peres et al., 2014).

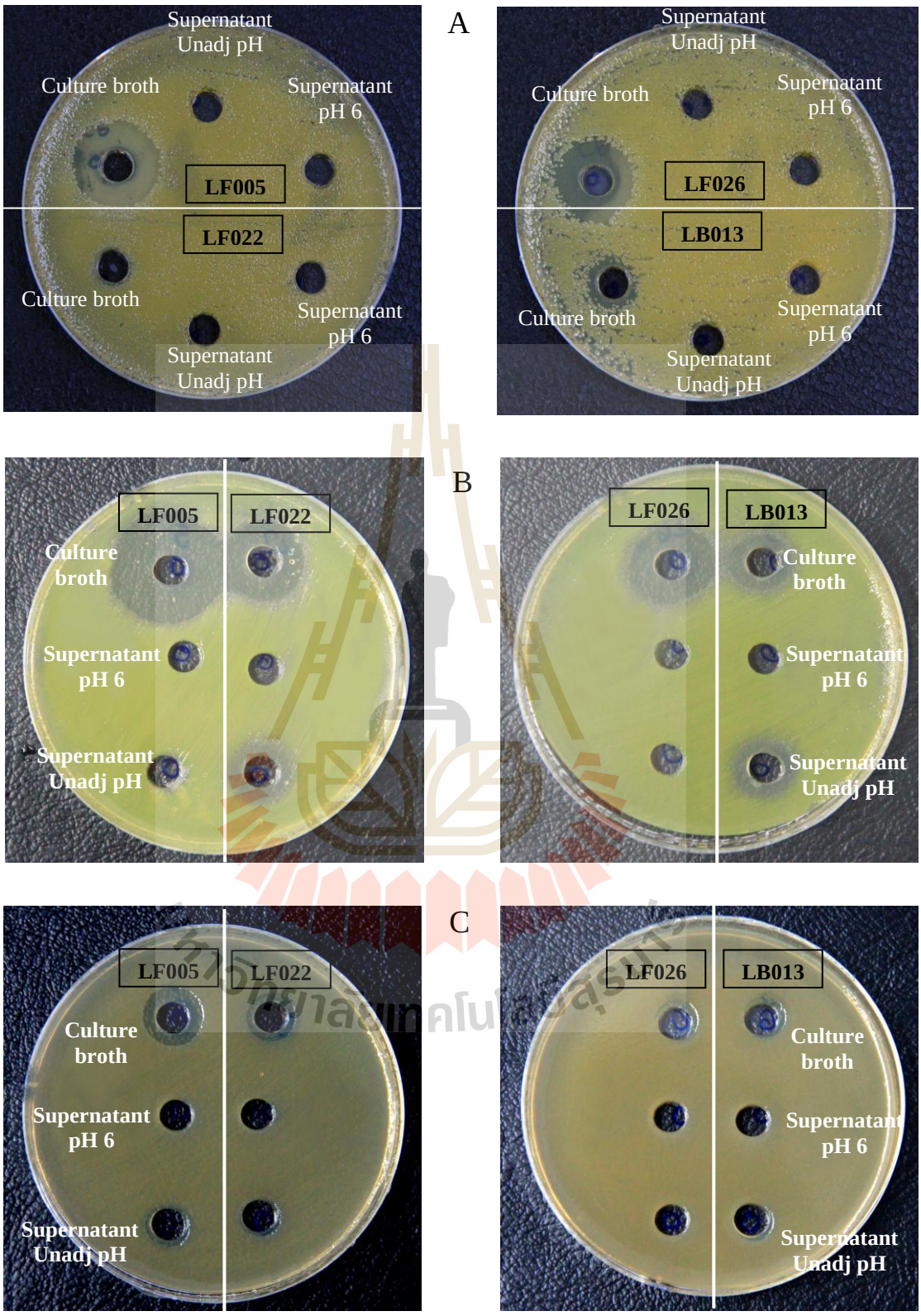


Figure 3.9 Inhibitory effects of lactobacilli against pathogenic bacteria; *S. aureus* (A), *P. aeruginosa* (B) and *V. cholerae* (C).

3.3.3 GI transit tolerance

Tolerance to digestive stress is one of the main factors limiting use of microorganisms as live probiotic agents, acid and bile salt tolerance are indeed considered essential properties required for probiotic be able to survive in the gut. (Peres et al., 2014; Solieri et al., 2014). The main purpose of the present study was to select the best probiotic strain for further development as an effective probiotic starter. This study mimics *in vivo* human GI passage which considered three relevant factors during digestion; the effect of lysozyme; the influence of acid pH values, together with pepsin and sequential gastric emptying at increasingly lower pH (reaching pH 2) and transit time of food through stomach and action of bile salts and pancreatin, coupled with sequential gastric delivery of bacteria to the intestine.

The results revealed that there were no differences ($p>0.05$) in any of their cell counts within the first 60 min of incubation when pH decreased from 6.0 to 3.0. When the simulated gastric juice reached pH 2.0, the reduction of viability of the candidate lactobacilli (LF005, LF022, LF026 and LB013) were approximately 4 log-units while BF052 showed a great resistance with a reduction of viability only 1 log CFU/mL. It was noted that a significant impact on survival of strains occurred only at pH 2 ($P>0.05$) which may thus be considered critical for selection of potential probiotic. The result also indicated that the majority of strains were more resistant to bile salts and pancreatic enzyme in the intestinal conditions than to low pH conditions.

In this study, only BF052 exhibited the highest survivability throughtout GI transit, suggesting that it may remain viable at levels necessary before adherence to the intestinal epithelium to exert health-promoting benefits there. Based

on the result obtained in this study, *B. animalis* BF052 was thus selected to develop as probiotic starter in the further study.

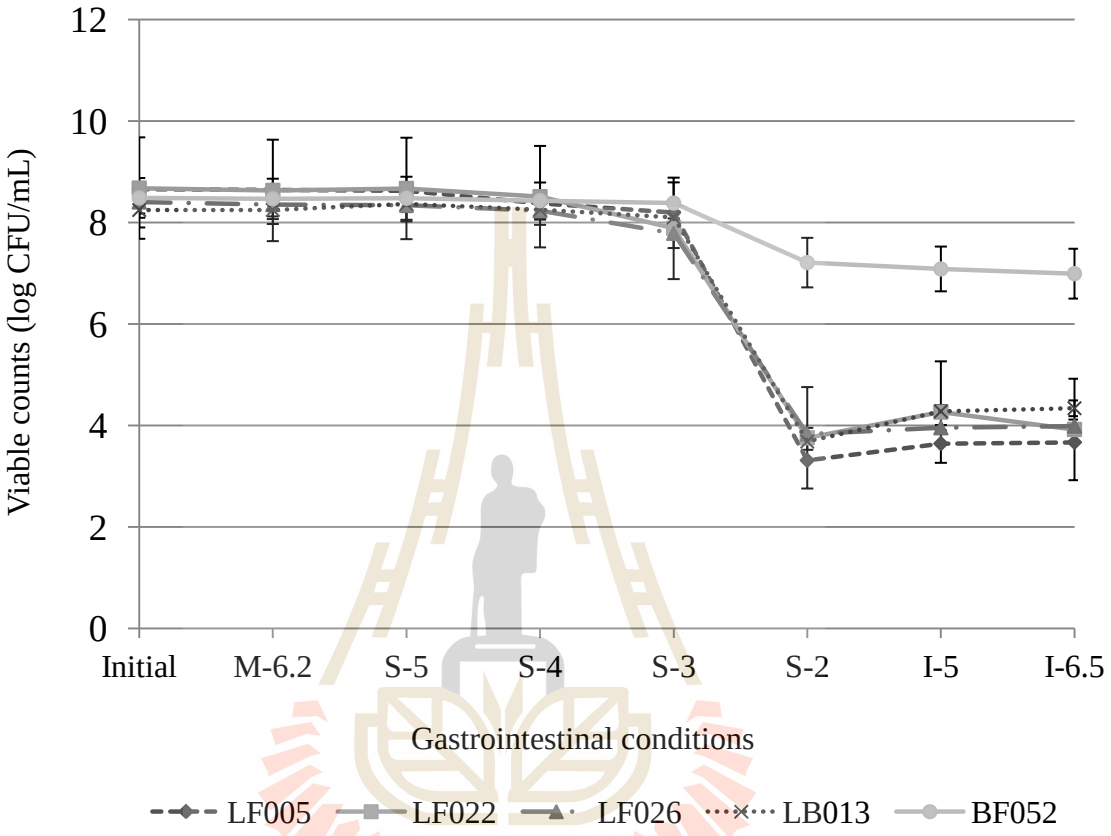


Figure 3.10 Viable cell numbers (log CFU/mL) at various stages of GI passage. (M-6.2; Mouth pH 6.2, S: Stomach conditions at pH 5, 4, 3 and 2, I: Intestinal conditions at pH 5 and 6.5)

3.3.4 Preservation of BF052 by freeze-drying in different cryoprotectants

In industrial applications, the use of probiotics as starter cultures is required to guarantee long-term delivery of stable cultures in terms of cell viability and functionality (Zhao and Zhang, 2005). Freeze-drying is a well-documented technique used for the preservation of microorganism (Khoramnia et al., 2011).

Moreover, the utilization of a suitable cryoprotective as a freeze-drying agent is an achievable attempt to improve cell viability during this process. In this study, sucrose, lactose, skim milk, GBR, BS, and soymilk were examined for their ability to protect the BF052 cells during freeze-drying. Table 3.14 shows the effect of cryoprotectants on the survivability of BF052 at different storage periods and temperatures.

Among all candidate cryoprotectants, only 10% skim milk showed no statistical difference ($p>0.05$) in protecting cells during freeze-drying. After storage, the survival rates of the BF052 freeze-dried cells were better at refrigerated temperatures than room temperature. It was reported that powdered *Bifidobacterium* preparations survived better in refrigerated storage than at room temperature (Saarela et al., 2006). In addition, there were no significant differences ($p<0.05$) in the viable cells using soymilk and BS after storage of freeze-dried powders for 1 month at refrigerated temperature and room temperature. However, after a month storage, the cell viability after 1 month storage in soymilk was higher than that of BS.

According to Carvalho et al. (2004), distinct properties of the cryoprotectants resulted in different protection features. The protective ability of skim milk on freeze-dried cells may be explained by its capacity in the prevention of cellular injury, stabilization of the cell membrane constituents, and provision of a protective coating for the cells (Huang et al., 2006). Vinderola et al. (2012) also found that skim milk and lactose were effective in the protection of *B. animalis* subsp. lactis INL1 comparable with sucrose during freeze-drying and storage, including after exposure under the harsh conditions of simulated digestion. Besides skim milk, soymilk is especially interesting as an attractive cryoprotectant. It is likely that soymilk contains many substances in protecting BF052 freeze-dried cells, such as

protein, which is equivalent to that of milk, soybean-oligosaccharides, stachyose, and raffinose. Moreover, it was reported that the survival of *B. animalis* subsp. lactis 10140 during the freeze-drying process was enhanced by the presence and increment of probiotics (Shamekhi et al., 2013). In contrast to skim milk and soymilk, GBR and BS are composed of mostly polymeric sugars. They easily form glasses that often do not have suitable structures to be able to depress membrane phase transition resulting in failure to protect microbial cells during the freeze-drying process (Santivarangkna et al., 2008). Zhao and Zhang (2005) suggested that a good cryoprotection should protect the cells during the freezing process, be easily dried, and provide a good matrix to allow stability and ease of rehydration. During rehydration using MRS broth, GBR and BS were not perfectly rehydrated due to complex substances and thus affected the survival rate of freeze-dried cells.

Nowadays, the demand for non-dairy probiotic products has increased and the use of soymilk as a cryoprotectant during the freeze-dried process is an option to develop a fully non-dairy probiotic product. However, dairy products are still the main vehicles for the incorporation of probiotic cultures (Pimentel et al., 2015b; Sharma et al., 2014). In this study, skim milk was the most effective protective agent for BF052 cells during freeze-drying and storage, and was therefore selected for further study.

Table 3.14 Effects of cryoprotective agents on cell survival of BF052 during freeze-drying (FD) and storage.

Cryoprotectants	Cell viability (log CFU/mL ± SD)		Cell viability after storage in refrigerator (log CFU/mL ± SD)			Cell viability after storage at room temperature (log CFU/mL ± SD)		
	Before FD	After FD	1 month	3 months	6 months	1 month	3 months	6 months
DI water	9.32 ± 0.07^{a}	$8.60 \pm 0.2\text{-}^{b}$	8.42 ± 0.02^{b}	8.10 ± 0.10^{c}	7.79 ± 0.04^{d}	6.09 ± 0.08^{e}	-	-
10% Sucrose	9.32 ± 0.27^{a}	9.02 ± 0.01^{b}	8.73 ± 0.01^{c}	8.68 ± 0.01^{cd}	8.44 ± 0.02^{d}	6.26 ± 0.19^{e}	$-^{\pi}$	-
10% Lactose	9.34 ± 0.22^{a}	8.99 ± 0.10^{b}	8.75 ± 0.02^{bc}	8.76 ± 0.08^{bc}	8.65 ± 0.04^{d}	7.80 ± 0.03^{e}	6.02 ± 0.16^{f}	3.10 ± 0.04^{g}
10% Skim milk	9.21 ± 0.07^{a}	9.16 ± 0.02^{a}	9.16 ± 0.01^{a}	9.15 ± 0.01^{a}	9.12 ± 0.01^{a}	8.78 ± 0.10^{b}	7.42 ± 0.08^{c}	6.06 ± 0.03^{d}
10% Germinated brown rice	9.81 ± 0.32^{a}	9.41 ± 0.23^{ab}	9.19 ± 0.12^{bc}	8.90 ± 0.10^{c}	nd	7.61 ± 0.19^{d}	5.14 ± 0.13^{e}	nd
10% Black sesame	9.36 ± 0.03^{a}	8.48 ± 0.65^{b}	8.39 ± 0.06^{b}	8.31 ± 0.08^{b}	nd	7.76 ± 0.16^{b}	6.58 ± 0.15^{c}	nd
Soymilk	9.20 ± 0.27^{a}	8.93 ± 0.10^{ab}	8.87 ± 0.09^{ab}	8.83 ± 0.09^{ab}	nd	8.77 ± 0.07^{ab}	7.33 ± 0.14^{c}	nd

- The equal superscript lowercase letters indicate no significant differences between cryoprotectant ($p>0.05$).

$^{\pi}$ No growth

nd Not determined

3.3.5 Effect of food processing chain on probiotic properties of BF052 during GI transit

This study aimed to examine the consistency of the probiotic properties of BF052 after the production process, including freeze-drying, storage, and incorporation of the strain into the products. After this process, BF052 was evaluated the tolerance ability through an *in vitro* model of the human GI tract. The strain was encountered the lysozyme-containing saliva in the mouth, pH gradient and gastric enzymes in the stomach, followed by the bile and pancreatic enzymes in the small intestine, and the adherence of the strain to human intestinal mucosa as a final step. Changes in cell viability by the end of each stage were examined.

Strain BF052 showed the ability to resist to the adverse conditions tested in every compartment as shown in Table 3.15. It exhibited a small susceptibility through each step, with different enzymatic- and pH-dependent barriers until gastric emptying at increasingly lower pH (reaching to pH 2.0). A significant reduction ($p \leq 0.05$) in cell survival occurred only at pH 2 in all processes. This strain was also resistant to the duodenum and ileum steps and retained its viability with a small reduction in viable counts. These results are consistent with those previously revealed from other *B. animalis* strains belonging to *B. animalis* BB-12 (Sousa et al., 2015) and *B. animalis* Bo (Madureira et al., 2011), which generally showed a great resistance throughout the whole processes of simulated digestion.

In addition, the impact of food manufacturing processes, such as freeze-drying, was also determined and compared with the direct adherence assay. The results showed that no significant differences ($P>0.05$) in adhesion capability

were detected among freeze-dried and non-freeze-dried cells. This result was in contrast to Du Toit et al. (2013) who reported that freeze-drying of probiotics was found to have an adverse effect on adhesion capability. Osmotic shock, formation of intracellular ice, and re-crystallization during freeze-drying may damage the biological structures of the cell and probably affect the adhesion ability of probiotics. However, use of an appropriate cryoprotectant during freeze-drying may reduce such adverse changes resulting in the maintenance of the ability of this strain to exhibit probiotic behavior (Jankovic et al., 2010). However, our experiments also demonstrated the effect of freeze-drying process on adhesion ability of probiotics after passage through the conditions of GI tract. Based on our results, it was observed that the introduction of BF052 through GI transit may enhance the adhesive ability to Caco-2 cells compared with those of non-challenged conditions. It may be explained that either acid or bile adaptation appeared to affect the *in vitro* adhesion to the intestinal cell line. Also, it was reported that the induction of acid or bile resistance in bifidobacteria may improve cellular surface properties and thus enhance the adhesion ability that favors their potential functionality as probiotics (Collado et al., 2006; Gueimonde et al., 2007; Sánchez et al., 2013).

Before delivering probiotic-containing products to consumers, probiotic bacteria should survive and retain their functionality not only during storage as freeze-dried cultures but also in the food products into which they are finally formulated (Saarela et al., 2000). Eventually, it would be beneficial that the strain would be supplemented into the pasteurized whole milk as one of the alternative means for delivering probiotics. In this study, after freeze-drying and the subsequent storage as freeze-dried powder for 1 month, BF052 were sequentially delivered in a

pasteurized whole milk as a probiotic vehicle and stored at refrigerated temperatures for two weeks. The survival of the strain throughout the process of simulated digestion was then monitored. Interestingly, the whole production process did not affect the stability of the probiotic properties of BF052, especially the resistance of this strain through GI transit, including adherence ability. BF052 still displayed a similar ability to withstand GI stresses and exhibited no significant variations ($P>0.05$) in adhesive ability to Caco-2 cells despite differences in cell preparations. Moreover, it was observed that carriers of probiotic bacteria were involved in affecting the viability and functionality of probiotics during storage and throughout the simulated GI system (Madureira et al., 2005). Kos et al. (2000) studied the effect of whey protein concentrate (WPC) on the viability of *L. acidophilus* M92, and found that addition of WPC may protect the cells from the low pH of simulated gastric juice, and even higher concentrations of bile salts. In addition, Madureira et al (2011) proposed that whey cheese matrices as a probiotic vehicle were shown to protect *L. casei*, *L. acidophilus*, and *B. animalis* during *in vitro* simulated digestion, compared with their performance in plain MRS medium. Saarela et al. (2006) also reported that acid and bile tolerances were better in freeze-dried *B. animalis* subsp. lactis E2010 added to pasteurized milk compared with those in phosphate-buffered saline or juice held at 4°C over two weeks. Therefore, several factors may influence the ability of the probiotics to survive in the product and become active when entering the consumer's GI tract. In this regard, the interactions of probiotics with the food matrix or the starter culture, pH, acidity, temperature, and oxygen content of the product are also important.

Probiotic strains selected for commercial application in foods must retain the characteristics for which they were originally selected (Sharma et al., 2014). In this report, even though the strains encountered potentially stressful conditions throughout the manufacturing processes and biological barriers during GI transit, BF052 still maintained its original characteristics. These included the characteristics of survival and tolerance during manufacture and after consumption, and during transit through the stomach and small intestine until adherence to the intestinal epithelium. Therefore, it is anticipated that BF052 retains its probiotic functionality and remains viable at levels necessary to provide health benefits to consumers. However, *in vivo* investigations are still necessary to fully validate its beneficial roles to the health of human hosts.

Table 3.15 Cell viability (log CFU/mL ± SD) within dynamic *in vitro* model and adhesion capability of *B. animalis* BF052 from different processes.

Process	Initial count	GI compartment								%Adhesion
		Mouth	Oesophagus-stomach					Duodenum	Ileum	
		2 min	pH 6 10 min	pH 5 10 min	pH 4 10 min	pH 3 30 min	pH 2 30 min	pH 5 30 min	pH 6.5 90 min	
BF052-Caco-2	8.13±0.26	-	-	-	-	-	-	-	-	3.19%±0.11^{A}
BF052-GI test-Caco-2	8.47±0.39^{a}	8.47±0.40^{a}	8.48±0.42^{a}	8.46±0.32^{a}	8.43±0.36^{a}	8.38±0.41^{a}	7.21±0.49^{b}	7.09±0.44^{b}	6.99 ± 0.49^{b}	3.81%± 0.32^{A}
BF052-FD-Caco-2	8.13±0.18	-	-	-	-	-	-	-	-	3.08%± 0.15^{A}
BF052 -FD-GI test-Caco-2	8.34±0.21^{a}	8.23±0.21^{a}	8.32±0.17^{a}	8.29±0.24^{a}	8.27±0.21^{a}	8.19±0.11^{a}	7.01±0.20^{b}	6.85±0.11^{b}	6.77 ± 0.18^{b}	3.45%±0.21^{A}
BF052-FD-milk-GI test-Caco-2*	8.53±0.21^{a}	8.49±0.20^{a}	8.50±0.24^{a}	8.48±0.23^{a}	8.43±0.2^{a}	8.39±0.27^{a}	7.45±0.14^{b}	7.35± 0.20^{b}	7.30 ± 0.19^{b}	3.67%±0.50^{A}

- The equal superscript lowercase letters in the same row indicate no significant differences between digestion steps ($p>0.05$).
- The equal superscript capital letter in the last column indicates no significant differences in adhesion percentage for each process ($p>0.05$).

* In this process, *B. animalis* BF052 were freeze-dried (FD) by using 10% skim milk as a cryoprotectant agent and then stored as freeze-dried powders for 1 month at refrigerated temperature following incorporation into a whole pasteurized milk and kept at refrigerated temperature for 2 weeks before exposure through GI (GI) transit followed by adherence assay (Caco-2).

3.4 Conclusion

It is crucial to investigate interesting strain characteristics in terms of safety and functional aspects for probiotic potential. In this regard, resistance of strains against production, storage, and GI tract stresses is of prime importance. This research demonstrated that *B. animalis* BF052 displayed promising probiotic properties and exhibited resilience to adverse conditions not only during industrial processes but also under GI environments before adherence to the intestinal epithelium to exert health-promoting benefits there. Therefore, *B. animalis* BF052 studied in this research is a potential probiotic candidate for further development as an effective probiotic starter.

CHAPTER IV

DEVELOPMENT OF FERMENTED SOYMILK SUPPLEMENTED WITH *BIFIDOBACTERIUM ANIMALIS* BF052

4.1 Introduction

Interest in health-promoting foods has recently increased among modern consumers due to a greater consciousness of wellness and nutritional perspectives. Better understanding of the role of probiotic bacteria in maintaining health of the host has resulted in a rapid increase of using these bacteria in different food products (Prasanna et al., 2014). It is generally know that probiotics products available in the markets today are mainly milk based. However, the ongoing trend of vegetarianism and a high prevalence of lactose intolerance have encouraged the research efforts targeted to develop probiotic foods outside the dairy section (Granato et al., 2010; Martins et al., 2013; Santos et al., 2014). Among the non-dairy probiotic products, soymilk based yogurt offers a considerable appeal for a growing segment with certain dietary and health concerns. In this regard, soymilk represents as the most inexpensive source with high-nutritional quality protein and several putative health-beneficial substances such as amino acids, vitamins, minerals, dietary fibre, isoflavones and other flavonoid compounds with strong antioxidant (Ma et al., 2015). The high nutritive values and health characteristics of soymilk encourage the consumer interest, however disagreeable beany flavors and indigestible oligosaccharides limit the

consumption of their usage. To overcome these limitations, fermentation with lactic acid bacteria and bifidobacteria has been attempted (Donkor et al., 2005; Farnworth et al., 2007; Champagne et al., 2009). Scalabrini et al. (1998) demonstrated that bifidobacteria were able to reduce α-galactosaccharides that can lead to flatulence and alkylic aldehydes that are responsible for a beany flavor. It was also proposed that fermentation of soymilk with mixed cultures offers not only a means of preserving soymilk but also a possibility for modifying or improving its flavor and texture as well as enhancing its beneficial health properties (Donkor et al., 2005; Li et al., 2014).

Our previously published works demonstrated that *B. animalis* BF052 possessed considerable probiotic properties, including high acid and bile tolerance, strong adhesion capability to Caco-2 cells, and inhibitory activity against pathogens including *Salmonella typhimurium* and *Vibrio cholerae* (Charnchai et al., 2016). Therefore, it is very beneficial to develop as a delivery medium for the live probiotic *B. animalis* BF052 that meets acceptable requirements to consumers. The aim of the present work was to investigate the behavior of the probiotic *B. animalis* BF052 in soymilk and the effects of this organism as part of the starter cultures along with the conventional starters, *Streptococcus thermophilus* and *Lactobacillus bulgaricus*. Microbial viability of starter cultures, physicochemical properties (pH and texture) and metabolic activities (lactic and acetic production) of fermented soymilk were determined during fermentation for 48 h. The sensory preferences of fermented soymilk prepared in combination with those starters were also evaluated to gain preliminary knowledge in product development. In addition, this work also presented the possibility in applying *B. animalis* BF052 as a probiotic starter in the production of fermented milk.

4.2 Materials and methods

4.2.1 Bacterial strains and culture conditions

The strains of *S. thermophilus*, *L. bulgaricus* and *B. animalis* BF052 were obtained from Suranaree University of Technology (SUT) culture collection, Thailand. The starter cultures were activated by growing 2 times successively at 37°C for 16 h in *Streptococcus thermophilus* (ST) broth (10 g/L casein enzymic hydrolysate, 5 g/L yeast extract, 10 g/L sucrose and 2 g/L K_2HPO_4) for *S. thermophilus* and in DeMan, Rogasa and Sharpe (MRS) broth (Oxoid Ltd., UK) for *L. bulgaricus*. For growing *B. animalis* BF052, MRS broth was supplemented with 0.05% L-cysteine-hydrochloride (Merck, Germany) (MRSc) and incubation under anaerobic condition at 37°C for 16 h. All strains were incubated and maintained in medium containing 20% (v/v) sterile glycerol and stored at -80°C.

4.2.2 Soymilk preparation

Whole soybeans were washed and soaked for 4-6 h in distilled water. After decanting the soaking water, the soybeans were then blended with distilled water. The resultant slurry was filtered through a double-layered cheesecloth to extract soymilk. Approximately 1,100 mL of soymilk was obtained from 250 g of soybeans in 1,500 mL of total distilled water. Soymilk was dispensed into containers and was sterilized by autoclaving at 110°C for 15 min.

4.2.3 Fermentation of soymilk

After two successive transfers at 37°C for 16 h in ST broth for *S. thermophilus*, MRS broth for *L. bulgaricus* and MRSc broth for *B. animalis* BF052,

cells were harvested (4,000 rpm, 5 min, 4°C) and washed twice with sterile distilled water before being re-suspended in soymilk to obtain initial population of each organism approximately 5-6 log CFU/mL. The starter cultures, *S. thermophilus* and *L. bulgaricus*, were used in single culture and/or in combination with *B. animalis* BF052 for the fermentation of soymilk. The cell suspension was then inoculated in a 150 ml screw-cap bottles containing 120 ml of sterile soymilk and incubated without shaking at 37°C for a period of 48 h. During fermentation, samples were collected at an interval of 12 h to determine the numbers of lactic acid bacteria and bifdobacteria, the pH, lactic and acetic acid contents and firmness of yogurt texture.

4.2.4 Determination of microbial viability

To determine the survival of *S. thermophilus*, appropriate dilutions in phosphate buffered saline (PBS; 0.08 g/L NaCl, 0.02 g/L KCl, 0.0144 g/L Na_2HPO_4 and 0.0024 g/L KH_2PO_4, pH 7.2) were plated on ST agar. Cell viability of *L. bulgaricus* and *B. animalis* BF052 was determined by plating on MRSc agar after 48-72 h of anaerobic incubation at 37°C.

4.2.5 Measurements of pH and lactic and acetic acid production

Changes in pH were monitored during fermentation of soymilk at 0, 12, 24, 36, and 48 h using a pH meter (pH 700 Benchtop Meter, Oakton, USA). Production of lactic and acetic acids was determined by high performance liquid chromatography (HPLC ; Agilent Technologies 1200, 2009) equipped with UV, refractive index detectors and an Aminex HPX-87H ion exclusion column (Bio-Rad Lab, U.S.A.). The mobile phase was 4 mM sulfuric acid with a flow rate set at 0.4

mL/min and the temperature of the column was set at 45°C. Two milliliters of fermented soymilk was withdrawn and centrifuged at 10,000 rpm for 10 min. The supernatant was then filtered through a 0.2-µm membrane filter before injecting to HPLC. Twenty microliters of injection volume were automatically analyzed through the HPLC system.

4.2.6 Determination of yogurt texture

Texture analysis was conducted as previously described by Mani-López et al. (2014). After fermentation, fermented soymilk was kept at refrigerated temperature for 24 h and then left at room temperature for 5 min before analysis. The experiment was carried out directly in a 150-mL sample jar using a texture analyzer TA.XT.plus (Stable Micro Systems, UK). A 60° cone probe (27 mm in diameter, 25 mm in height and weight of 18.144 g) was moved at a test speed of 10 mm/s from the fermented soymilk surface until a distance of 20 mm within the sample was reached. This test quantified the gel strength (firmness) by the positive area of the graph of force (N) versus time (s), which indicated the strength to break it.

4.2.7 Sensory evaluation

The sensory preferences of fermented soymilk prepared in conjunction with *S. thermophilus*, *L. bulgaricus* and *B. animalis* BF052 were evaluated by 30 untrained panelists. Fermented soymilk terminated at 12 h, 16 h, 20 h and 24 h of fermentation time was scored in term of appearance, odor, texture, taste and overall acceptability through a hedonic 9-point scale in which; 9 = “like extremely”, 5 = “neither like nor dislike” and 1 = “dislike extremely”. The samples in 150 ml screw-cap bottle were kept at 4°C and conditioned at room temperature for 15 min before

testing. Sensory evaluations were privately conducted while participants were seated in a quite area. Each panelist was given a pen for recording on her/his evaluation sheet. A glass of water and unsalted crackers were provided to the panelists to cleanse their palate during tastings.

Before sensory evaluation, each sample in different terminated time was taken to measure the changes in fermented soymilk including cell viability, pH, lactic and acetic contents, firmness and syneresis. To determine syneresis, a tube containing 3 mL of fermented soymilk was weighed and centrifuged at 10,000 rpm for 10 min. After centrifugation, the separated supernatant was then weighed. Syneresis was calculated using the following equation; Syneresis (%) = $(W_s)(100) / W_{fs}$; where W_s was the supernatant weight after centrifugation and W_{fs} was the weight of the fermented soymilk in the tube.

4.2.8 Production of fermented milk

An inoculum of *S. thermophilus*, *L. bulgaricus* and *B. animalis* BF052 cultures was aseptically distributed into 120 mL portion of commercial pasteurized milk to obtain a final concentration of 5-6 log CFU/mL. Cell counts, pH change, measurements of lactose reduction and acetic and lactic production were determined at an interval of 6 h during fermentation at 37°C for 24 h.

4.2.9 Statistical analysis

Data were analyzed using SPSS 16.0 software (SPSS Inc., Chicago, IL, USA). Statistical differences in multiple groups were determined by one-way analysis of variance (ANOVA) followed by multiple mean comparisons with Duncan's test.

All numerical data were displayed as mean ± standard deviation and $p \leq 0.05$ was considered statistically significant.

4.3 Results and discussion

4.3.1 Growth behaviors during the fermentation of soymilk

Cell growth of lactic acid bacteria and bifidobacteria in soymilk during fermentation for 48 h is shown in Table 4.1. In this study, cell growth was monitored by enumurating viable cells on appropriated media (Figure 5.1). The results revealed that soymilk could support the growth of *S. thermophilus*, *L. bulgaricus* and *B. animalis* BF052. All strains were capable of growing in soymilk with no additives as high as 10^9 CFU/mL. During fermentation with single culture, *S. thermophilus* grew rapidly with the highest viable count while *L. bulgaricus* and *B. animalis* BF052 showed a slight decline in cell numbers at 48 h of fermentation.

It was reported that mixed cultures with *S. thermophilus* in fermented soymilk reduced populations of probiotic bifidobacteria (Champagne et al., 2009). In this study, no significant differences ($p>0.05$) were observed in cell counts either fermented with single culture of *B. animalis* BF052 or in combination with *S. thermophilus*. After incubation for 24 h, Wang et al (2002) revealed that the numbers of *Bifidobacterium infantis* and *Bifidobacterium longum* in the mixed culture with *L. bulgaricus* were higher than single culture. Farnworth et al (2007) also stated that the growth of bifidobacteria in soymilk could enhance by the liberation of nutrients, such as amino acids from other bacteria. After the start of the fermentation, time was thus required to generate enough amino acids to support the growth of probiotic bifidobacteria. This result was in accordance with the present study's

finding that the cell counts of *B. animalis* BF052 in soymilk fermented with *L. bulgaricus* were significantly higher ($p \leq 0.05$) than the numbers in pure culture. In contrast, the viable cells of *L. bulgaricus* were slightly decreased after fermentation for 24 h comparable with single culture. Wang et al. (2002) demonstrated that bifidobacteria exert a detrimental effect on the growth of *L. bulgaricus* in soymilk. This effect may be attributed to acetic acid accumulation in fermented soymilk by bifidobacteria.

It was proposed that the proteolytic rod, *L. bulgaricus*, enhances the growth of the streptococci in milk by producing small peptides and amino acids. On the other hand, *S. thermophilus* supports the growth of *L. bulgaricus* by the formation of formic acid from pyruvic acid under anaerobic conditions. As similar to what was observed in milk, in this study, a cooperative relationship with respect to viable cells was also observed in soymilk between *S. thermophilus* and *L. bulgaricus*. During the first 12 h of cultivation, approximately 1 log increases in the numbers of *S. thermophilus* and *L. bulgaricus* were observed when both starters were added together. This result was in line with previous study which showed some of the symbiotic elements of the relationship by these two yogurt starters might occur in the soy beverage also (Farnworth et al., 2007). Moreover, the numbers of *B. animalis* BF052 cells in soymilk fermented with those starter cultures were also significantly higher ($p \leq 0.05$) comparable with pure culture. Despite cultivation in conjunction with three strains, the growth of *L. bulgaricus* and *B. animalis* BF052 was more extensive than those obtained with single strain or in combination with two cultures. At the end of fermentation, the final numbers of each strain were also presented at a level of over 10^9 CFU/mL. However, the accumulation of organic acids during fermentation may

cause a slight reduction in the numbers of *S. thermophilus* when the fermentation time was extended to 48 h.

Table 4.1 Viable cell counts (log CFU/mL) in soymilk during fermentation for 48 h.

Strain	Fermentation time (h)				
	0	**12**	**24**	**36**	**48**
1.ST	5.54±0.06^{a}	9.72±0.06^{c}	10.03±0.13^{abc}	11.93±0.31^{c}	12.19±0.09^{d}
2.LB	5.56±0.01^{a}	8.60±0.26^{a}	9.78±0.06^{abc}	10.45±0.33^{b}	9.94±0.34^{ab}
3.52	5.67±0.45^{a}	8.55±0.16^{a}	9.56±0.22^{ab}	10.14±0.60^{ab}	9.99±0.89^{ab}
4.ST+ 52					
ST	5.46±0.21^{a}	10.36±0.53^{d}	10.73±0.17^{cd}	10.58±0.25^{b}	10.34±0.24^{ab}
52	5.67±0.07^{a}	8.69±0.27^{a}	9.47±0.38^{ab}	9.79±0.28^{ab}	10.41±0.03^{ab}
5.LB+52					
LB	6.10±0.66^{a}	8.74±0.14^{a}	9.19±0.03^{a}	9.39±0.08^{a}	9.59±0.03^{a}
52	6.08±0.33^{a}	8.87±0.09^{ab}	10.04±1.11^{abc}	10.57±0.48^{b}	10.69±0.20^{bc}
6.ST+LB					
ST	5.43±0.09^{a}	11.54±0.02^{f}	11.83±0.51^{e}	11.53±0.15^{c}	11.35±0.56^{cd}
LB	5.42±0.10^{a}	9.30±0.21^{bc}	9.57±0.36^{ab}	9.36±0.12^{a}	9.50±0.04^{a}
7.ST+LB+52					
ST	5.56±0.05^{a}	10.91±0.09^{e}	11.26±0.06^{de}	11.55±0.28^{c}	11.53±0.19^{cd}
LB	6.12±0.62^{a}	9.44±0.02^{c}	9.93±0.55^{abc}	10.10±0.52^{ab}	10.16±0.58^{ab}
52	6.12±0.78^{a}	9.56±0.02^{c}	10.42±0.38^{bcd}	10.34±0.31^{b}	10.86±0.18^{bc}

- ST: *S. thermophillus*, LB: *L. bulgaricus*, 52: *B. animalis* BF052

- The equal superscript lowercase letters in the same column indicate no significant differences in the fermentation time ($p>0.05$).

Champagne et al. (2009) noted that the development of fermented products containing probiotics require the ability of the promising strain to grow in the substrate as well as ability to compete or even establish a synergy between strains. In the present study, the growth behavior of *B. animalis* BF052 prepared with commercial yogurt starters fulfills these preliminary requirements with respect to high viable cell numbers throughout the fermentation period. This result indicated that

B. animalis BF052 could be supplemented as a probiotic starter that employ soymilk as the substrate.

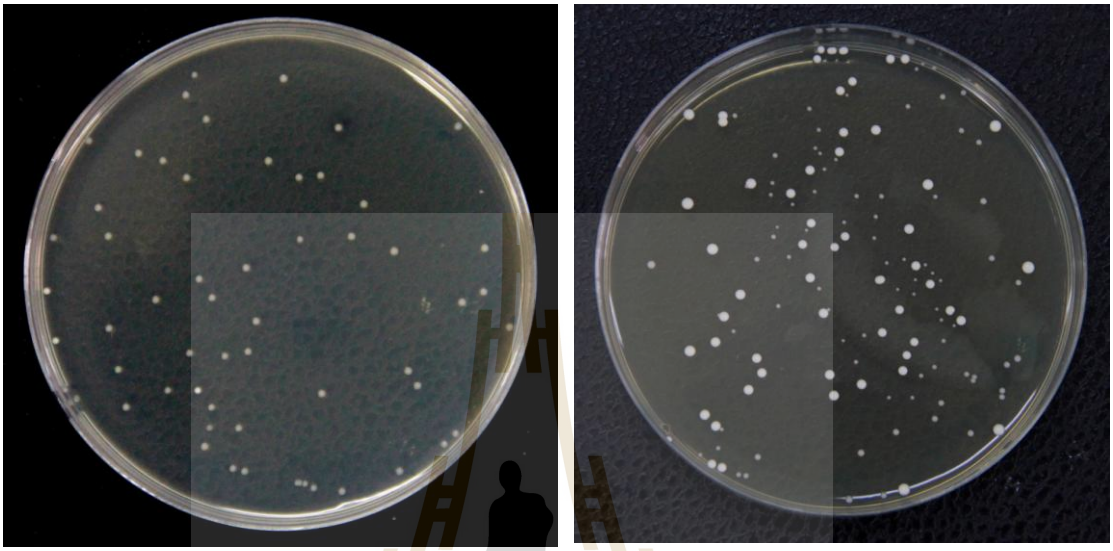


Figure 4.1 Colony morphology of *S. thermophilus* on ST agar (Left) and *L. bulgaricus* and *B. animalis* BF052 (tiny colonies) on MRSc agar (Right).

4.3.2 Changes in pH during fermentation

Figure 4.2 shows the changes in pH of soymilk during fermentation for 48 h. The pH changes of each strain appeared to be strain-dependent. A major significant reduction ($p \leq 0.05$) in pH was noted in soymilk fermented with single culture of *S. thermophilus* whereas the lowest change was found in soymilk inoculated with *L. bulgaricus*. This result was in accordance with Wang et al. (2002) that showed the highest pH found in soymilk fermented with *L. bulgaricus* (pH 5.97) and *B. longum* (pH 6.02) comparable to *S. thermophilus* (pH 3.84) after fermentation for 48 h. During fermentation with mixed cultures, pH values of soymilk prepared in

combination with *S. thermophilus* also declined significantly ($p \leq 0.05$), especially during the first 12 h of cultivation. Generally, fermentation was carried out until a pH of 4.5 was reached. As a result, the use of probiotic cultures requires a large fermentation period to reach low pH values (Mani-López et al., 2014; Santos et al., 2014). In this study, the shortest period of fermentation could be obtained in soymilk fermented with the mixed cultures of *B. animalis* BF052 along with *S. thermophilus* and *L. bulgaricus*. The reduction of pH values during fermentation indicates a greater rate of production of organic acids in fermented soymilk.

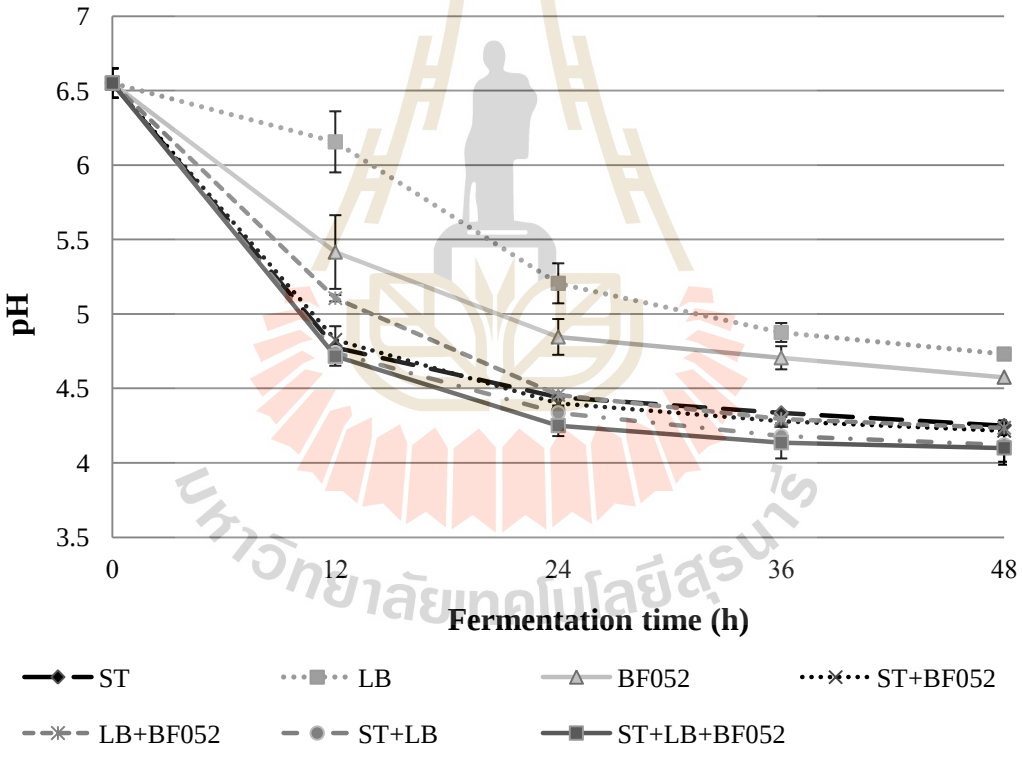


Figure 4.2 pH profiles during soymilk fermentation.

4.3.3 Changes of lactic and acetic acids during fermentation

The concentration of lactic and acetic acids in fermented soymilk prepared with lactic acid bacteria or simultaneously with bifidobacteria is shown in

Figure 4.3. Regardless of the culture strains, the amounts of acetic and lactic acids increased in soymilk when fermentation period was extended. It was observed that single culture of *S. thermophilus* produced lactic acid as a major organic acid. At 12 h of fermentation with single culture, the highest amount of lactic acid of 3.58±0.78 g/L was observed in soymilk fermented with *S. thermophilus* compared with those of 0.47±0.03 and 0.48±0.06 g/L in soymilk fermented with *L. bulgaricus*, and *B. animalis* BF052, respectively. Then, the concentration of lactic acid in *S. thermophilus* and *L. bulgaricus* increased consistently until 48 h of incubation. However, after 24 h of fermentation, no significant differences ($p>0.05$) were observed in lactic acid content in soymilk prepared with *B. animalis* BF052. The final concentration of lactic acid produced by *B. animalis* BF052 was found only 1.97±0.35 g/L. Since *S. thermophilus* and *L. bulgaricus* could produce lactic acid as a major fermentative product, the production of lactic acid in soymilk prepared in conjunction with *S. thermophilus-L. bulgaricus* and *S. thermophilus-L. bulgaricus-B.animalis* BF052 after 24 h of fermentation was thus significantly ($P<0.05$) higher than other combinations.

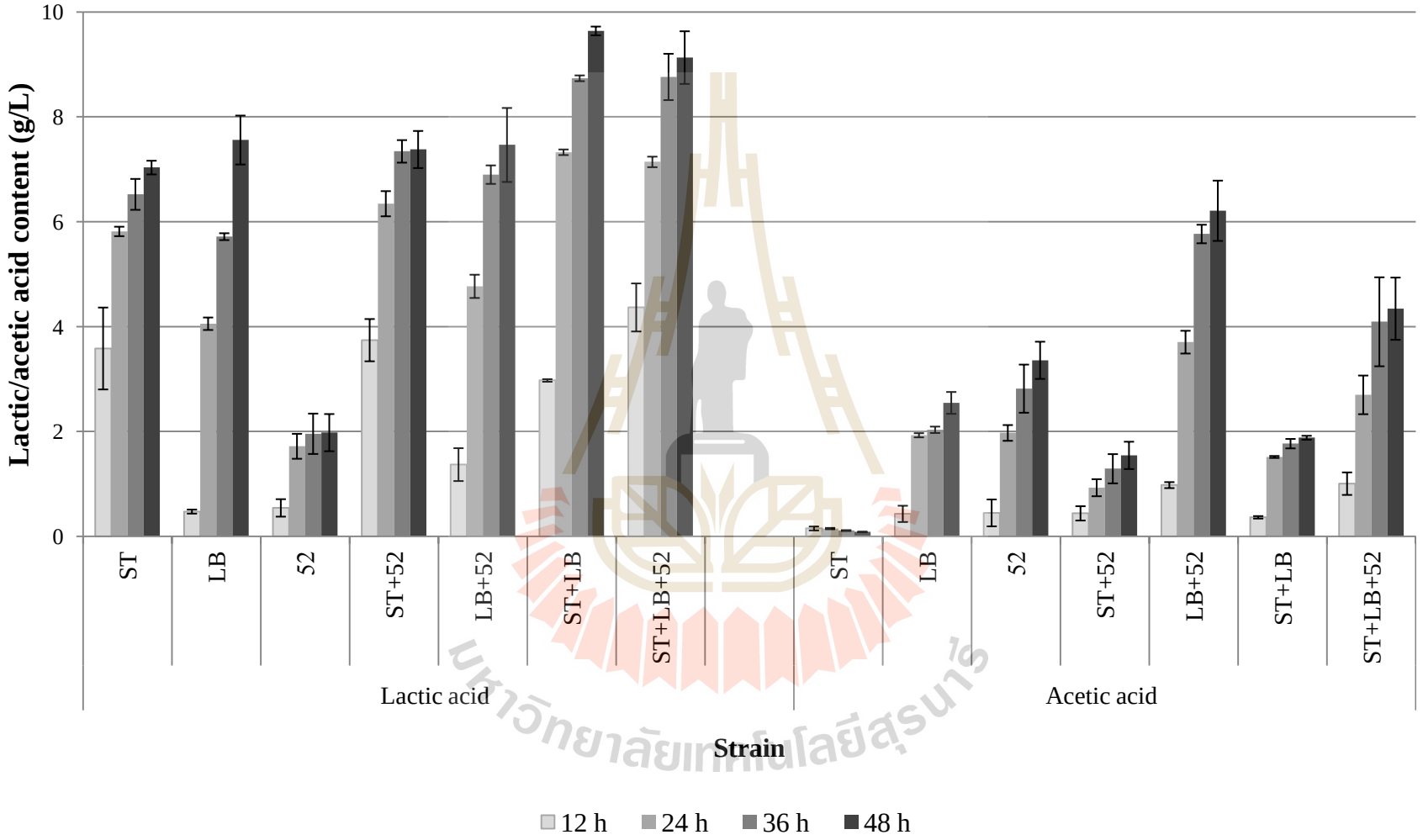


Figure 4.3 Changes in acetic acid and lactic acid concentrations (g/L) in fermented soymilk.

In contrast to the production of lactic acid, *S. thermophilus* produced the lowest concentration of acetic acid ranging from 0.08±0.01-0.15±0.01 g/L whereas *L. bulgaricus* (0.42±0.15-2.55±0.21) and *B. animalis* BF052 (0.33±0.06-3.36±0.35) produced a relatively higher amount of acetic acid during fermentation for 48 h. Therefore, introduction of bifidobacteria with *L. bulgaricus* resulted in a higher content of acetic acid than that of the other cultivation. Theoretically, metabolism of carbohydrates by bifidobacteria may lead to mainly production of acetic and lactic acid in the molar ratio of 1.5 (Wang et al., 2003). Hou et al. (2000) reported that the molar ratios of acetate/lactate in soymilk during fermentation for 48 h varied between 1.45-1.92 and 1.48- 1.84 for *B. infantis* CCRC 14633 and *B. longum* B6, respectively. They also demonstrated that although acetic and lactic acid contents increased during fermentation, the molar ratio of acetic and lactic decreased. This observation was in contrast to the current study which found that ratio of acetic and lactic acids increased until the end of fermentation. The molar ratio was found to be 0.82, 1.15, 1.44, 1.70 g/L at 12, 24, 36 and 48 h of incubation, respectively. These results suggested that capability to produce acetic and lactic varied with strains. It is generally known that stachyose and raffinose are the principal oligosaccharides in soymilk. Therefore, the different ability to utilize these α-D-galactosyl oligosaccharides by α-galactosidase enzyme may result in the different amounts of organic acids presented in soymilk (Donkor et al., 2007; Santos et al., 2014).

4.3.4 Textural characteristics

Textural profiles of fermented soymilk prepared either individually or in combinations with commercial starters were determined during fermentation for 48 h. Different types of the culture starters including the extension of fermentation

time contribute to different trends of firmness as presented in Figure 4.4. Damin et al. (2008) reported firmness values ranging from 0.64 to 0.93 N were observed in yogurts prepared with *S. thermophilus-L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus-L. acidophilus*. In this study, firmness values were in the range of 0.31±0.01-0.62±0.03 N for soymilk fermented with single culture or with the mixed cultures of *S. thermophilus-B.animalis* BF052 and *L. bulgaricus-B.animalis* BF052. However, the firmness of soymilk prepared in conjunction with *S. thermophilus-L. bulgaricus* (0.81±0.01-1.22±0.15 N) and *S. thermophilus-L. bulgaricus-B.animalis* BF052 (1.00±0.09-1.10±0.12 N) were significantly improved ($p \leq 0.05$) comparable with the others at all periods of the fermentation. Figure 4.5 illustrated the measurement of firmness of fermented soymilk by texture analyzer.

At 12 h of incubation, a yogurt texture was observed in soymilk prepared with *S. thermophilus* alone or in combination with *S. thermophilus* while other starters formed yogurt-like structures within 24 h of fermentation time (data not shown). Therefore, it seemed that *S. thermophilus* was responsible for lowering pH and thus producing the semisolid texture of yogurt during the first 12 h of fermentation time. However, the higher firmness was noted only in the presence of co-fermentation of *S. thermophilus* and *L. bulgaricus*. Interestingly, firmness also exhibited high values in soymilk prepared in conjunction with those starters and *B.animalis* BF052. Sah et al. (2016) demonstrated that lower firmness might result from larger pores in the gel networks, thus reduced cross-linking between molecules and caused a weak gel. In addition, Casarotti et al. (2016) also suggested that formation of a weak gel might involve in an acidification rate during the development of protein networks.

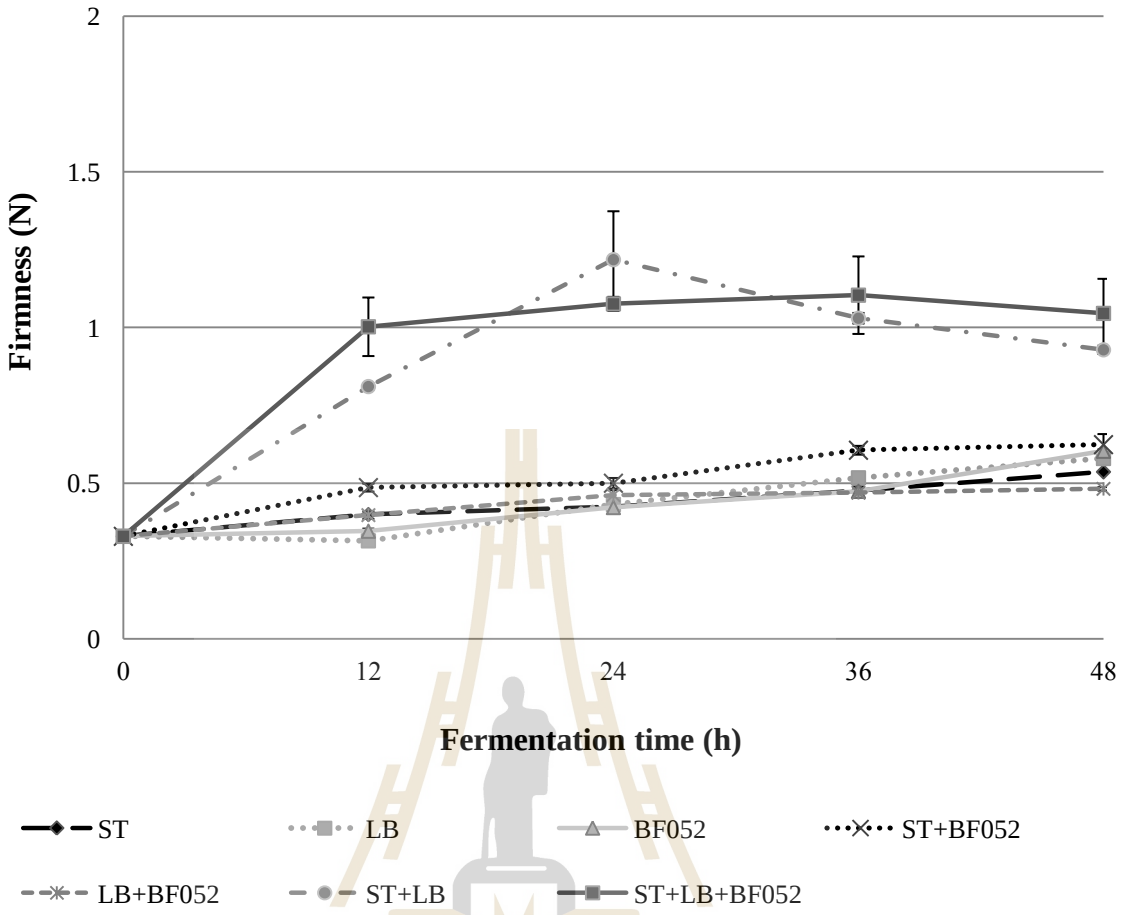


Figure 4.4 Firmness profiles of fermented soymilk.

During fermentation, pH of soymilk was reduced due to organic acid production, which caused the pH approach to the isoelectric point of the protein. This made the protein changed its state to gel and contained water in the protein gel network resulting in the increment of soybean curd yield (Jianming et al., 2013). In general, a higher firmness has been related to a longer fermentation time. However, the extension of fermentation time might lead to structural rearrangements and therefore lower gel stability and higher levels of syneresis (Sah et al., 2016). In this study, soymilk fermented with *S. thermophilus-L. bulgaricus* showed a significant

reduction ($p \leq 0.05$) in firmness after 24 h of cultivation while no significant difference ($p > 0.05$) was observed in *S. thermophilus-L. bulgaricus-B.animalis* BF052 throughout the fermentation period. To the best of our knowledge, the textural profiles of fermented soymilk, which was represented by the firmness value, were affected not only by the extension of fermentation time but also the combination of strain used.

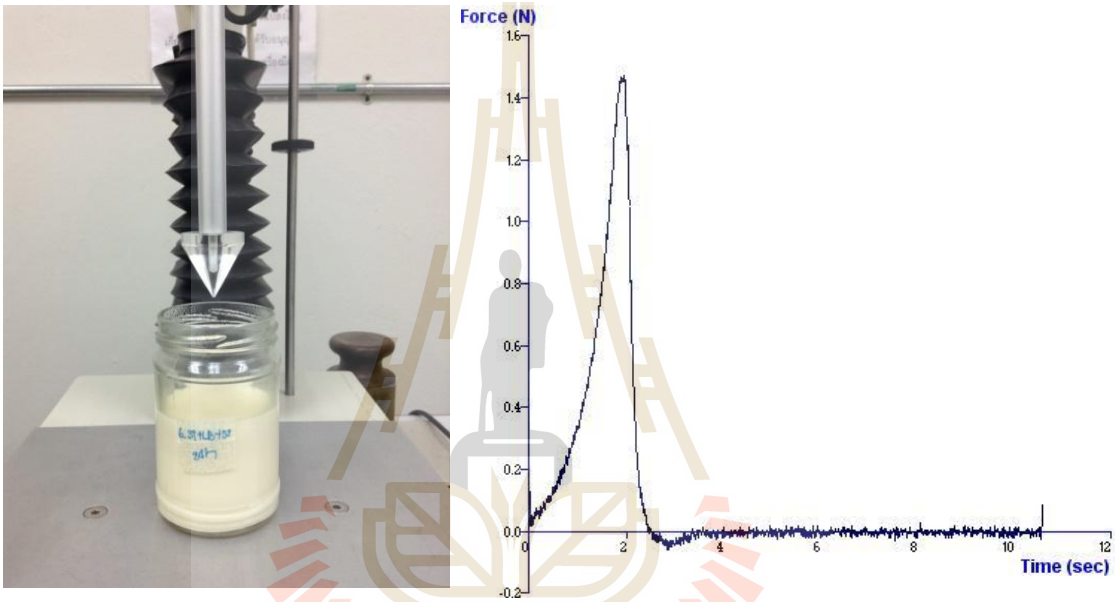


Figure 4.5 Measurement of firmness of fermented soymilk by texture analyzer (Left) and the graph of force (N) versus time (s) after measurement (Right).

Based on viability and physicochemical results, co-cultures of *S. thermophilus*, *L. bulgaricus* and *B. animalis* BF052 showed consistent increases in cell concentration from the start of incubation to reach the pH of 4.5 within 24 h. In addition, texture of fermented soymilk prepared in conjunction with these starters also exhibited constant firmness values throughout fermentation. Therefore, soymilk fermented in conjunction with those organisms during 12-24 h was then prepared for studying sensory evaluation.

4.3.5 Sensory evaluation of fermented soymilk

The sensory preferences of fermented soymilk prepared with commercial starters and *B. animalis* BF052 with different collected time were evaluated by 30 untrained panelists through a hedonic 9-point scale. The results revealed that there were no significant differences ($p>0.05$) in the overall preferences in term of appearance, odor, texture, taste and overall acceptability between fermented soymilk collected at 12 h and 16 h of fermentation (Table 4.2). The appearance, taste and overall acceptability were scored as the best in fermented soymilk harvested at 16 h whereas the 12 h fermentation had the highest score for odor and texture. However, soymilk fermented for 24 h had the lowest score for all appreciation. This result indicated that the evaluation scores decreased when fermentation period extended.

Table 4.2 Sensory preferences of fermented soymilk prepared in combination with *S. thermophilus*, *L. bulgaricus* and *B. animalis* BF052 at different harvest time.

Characteristic	Fermentation time (h)			
	12	16	20	24
Appearance	6.57 ± 1.59^{a}	6.87 ± 1.72^{a}	6.37 ± 1.88^{a}	4.93 ± 1.85^{b}
Odor	6.27 ± 1.87^{a}	5.67 ± 2.26^{a}	4.50 ± 2.08^{b}	3.60 ± 1.61^{b}
Texture	5.93 ± 1.78^{a}	5.83 ± 1.68^{a}	5.30 ± 2.04^{ab}	4.67 ± 1.84^{b}
Taste	5.27 ± 1.85^{a}	5.40 ± 2.21^{a}	4.07 ± 2.13^{b}	3.43 ± 1.63^{b}
Overall acceptability	6.03 ± 1.65^{a}	6.23 ± 1.78^{a}	4.97 ± 1.79^{b}	4.23 ± 1.67^{b}

- The equal superscript lowercase letters in the same row indicate no significant differences in consumers' preferences ($p>0.05$).

According to the consumers' preferences of the products, it was possible to infer some relationships between instrumental and sensory measurements. During fermentation, the characteristic aroma and flavor of fermented soymilk were released through metabolic processes by respective microorganisms. In general, short fermentation may have a mild aroma and flavor due to small amount of organic acids. It was noted that high amount of acetic acid is an undesirable product in fermented soymilk due to its vinegary flavor (Donkor and Shah, 2008). Interestingly, the co-cultivation of three cultures in this study showed low acetate production although all strains grew well and reached populations above 9 log CFU/mL since 12 h of incubation (Table 4.3). Approximately 1 g/L of acetic acid gradually increased from 1.20±0.06 to 3.44±0.45 during interval of incubation while the concentration of lactic acid in soymilk increased consistently from 3.40±0.62 to 7.31±0.24 during 12-24 h of incubation. This was in accordance with Donkor et al. (2007) who reported that some starters (*Bifidobacterium lactis* B94, *B. longum* Bl536, *L. delbrueckii* subsp. bulgaricus Lb1466 and *S. thermophilus* St1342) produced lower amounts of organic acids in soymilk even though they grew well. Hui and Özgül (2012) stated that although bifidobacteria produce acetic acid, representing an off-flavor in fermented soymilk, this effect can diminish with fermentation using mixed cultures with the lactobacilli and bifidobacteria. It was suggested that the mixtures of lactobacilli and bifidobacteria provided better result in the balance of acidity and conferred a more acceptable product. Moreover, it was also reported that the presence of *Bifidobacterium* could reduce the unpleasant off-flavour of n-hexanal and pentanal compounds responsible for the bean flavor in soy products (Farnworth et al., 2007; Scalabrini et al., 1998). In the present study, odor attribute of soymilk fermented for

12 h was the most preferable by the panelists, this was probably due to its low acidity and mild aroma. In case of taste of the product, although extension of fermentation time to 16 h resulted in pH reduction to 4.39 and higher amounts of organic acids, the sensory scores still showed no significant differences (P>0.05). This result indicated that metabolic and physiochemical changes by time extension during 12-16 h were not affected to sensory preferences evaluated by the consumers.

Table 4.3 Changes in microbial and physicochemical properties of fermented soymilk prepared for sensory evaluation.

Parameter	**Fermentation time (h)**			
	12	**16**	**20**	**24**
Cell growth (log CFU/mL)				
-*S. thermophilus*	11.64±0.08^{bc}	11.84±0.02^{bc}	11.93±0.01^{c}	11.54±0.17^{bc}
- *L. bulgaricus*	9.04±0.11^{a}	9.19 ±0.33^{a}	9.24±0.29^{a}	9.25±0.08^{a}
- *B. animalis* BF052	11.22±0.72^{b}	11.68±0.16^{bc}	11.87±0.01^{c}	11.64±0.08^{bc}
Lactic acid (g/L)	3.40±0.62^{a}	5.37±0.01^{b}	6.84±1.04^{bc}	7.31±0.24^{c}
Acetic acid (g/L)	1.20±0.06^{a}	2.10±0.08^{ab}	3.16±0.78^{bc}	3.44±0.45^{c}
pH	4.59±0.02^{c}	4.39±0.01^{b}	4.25±0.02^{a}	4.19±0.04^{a}
Firmness (N)	1.16±0.01^{b}	1.23±0.04^{c}	1.15±0.01^{b}	1.08±0.01^{a}
Syneresis (%)	20.43±0.52^{a}	19.55±0.84^{a}	22.82±0.42^{b}	23.96±0.55^{b}

- The equal superscript lowercase letters in the same row indicate no significant differences in each parameters (p>0.05).

Hwang and Hong, (2013) and Majchrzak et al. (2010) stated that the appearance characteristic of fermented soymilk generally refers to the homogeneity of the products and/or the presence of any components such as grains or water including

the intensity of the color that are separately visible. Degree of uniformity of particles analyzed with a spoon or perceived inside the mouth is referred as a texture. In our study, there were no significant differences ($p>0.05$) in the appearance and texture between fermented soymilk collected at 12 h and 16 h of fermentation. Sah et al. (2016) revealed that a syneresis is a major visible defect, appearing as separation of the liquid phase on the surface of yogurt gels. During growth, pH reduction causes rearrangement of the protein network resulting in loss of yogurt gel ability to entrap water phase and thus weakening of gel structure. This change leads to an increase in the number of particle-particle junctions and causes the gel to retract, expelling its interstitial fluid (Casarotti et al., 2014). As a result, it adversely affects the consumer acceptability of product not only the appearance characteristic but also the texture of product. Based on our result, a significant increase ($p\leq0.05$) in syneresis was observed after extension time to 20 h and probably had an impact on the reduction of sensory preferences. Therefore, the extending the fermentation time not only increased the fermentation cycle resulting in accumulation of fermentation products and changed the physicochemical properties of the soymilk but considerably affected the organoleptic properties of the product also.

It is generally known that short fermentation period was preferred by food manufacturing industry (Santos et al., 2014). In this study, mixed culture fermentations with *B. animalis* BF052 provide synergistic growth between strains and require short fermentation time during 12-16 h to reach the optimal levels necessary to provide health benefits and to achieve the best acceptable preferences. However, there was no addition of sugar and stabilizers during manufacturing process of soymilk in this report, further studies in product development should be conducted to enhance

consumer acceptability. This study presents relevant information on microbial, physicochemical and sensory properties of probiotic soy yogurt which could provide preliminary guidelines for developing a new probiotic-containing product.

4.3.6 Possibility of applying *B. animalis* BF052 as probiotic starter in fermented milk

It was reported that most strains of *Bifidobacterium* grow slowly in milk due to their poor proteolytic activity, which hinders their possible application as starter culture in fermented milk products (Prasanna et al., 2014). The results in the present study indicated that milk could not support the growth of *B. animalis* BF052. No growth of *B. animalis* BF052 was observed in fermented milk prepared with the ordinary starters, *S. thermophilus* and *L. bulgaricus*, during fermentation of milk for 24 h (Figure 4.6A). In other words, no significant changes ($p>0.05$) in the concentration of lactose, lactic acid and acetic acid were detected in fermented milk prepared with the single culture of *B. animalis* BF052 throughout the fermentation (Table 4.4). This observation was contrast to the result obtained from soymilk fermented with those commercial starter and *B. animalis* BF052, which showed high viable cell numbers throughout the incubation period (Figure 4.6B).

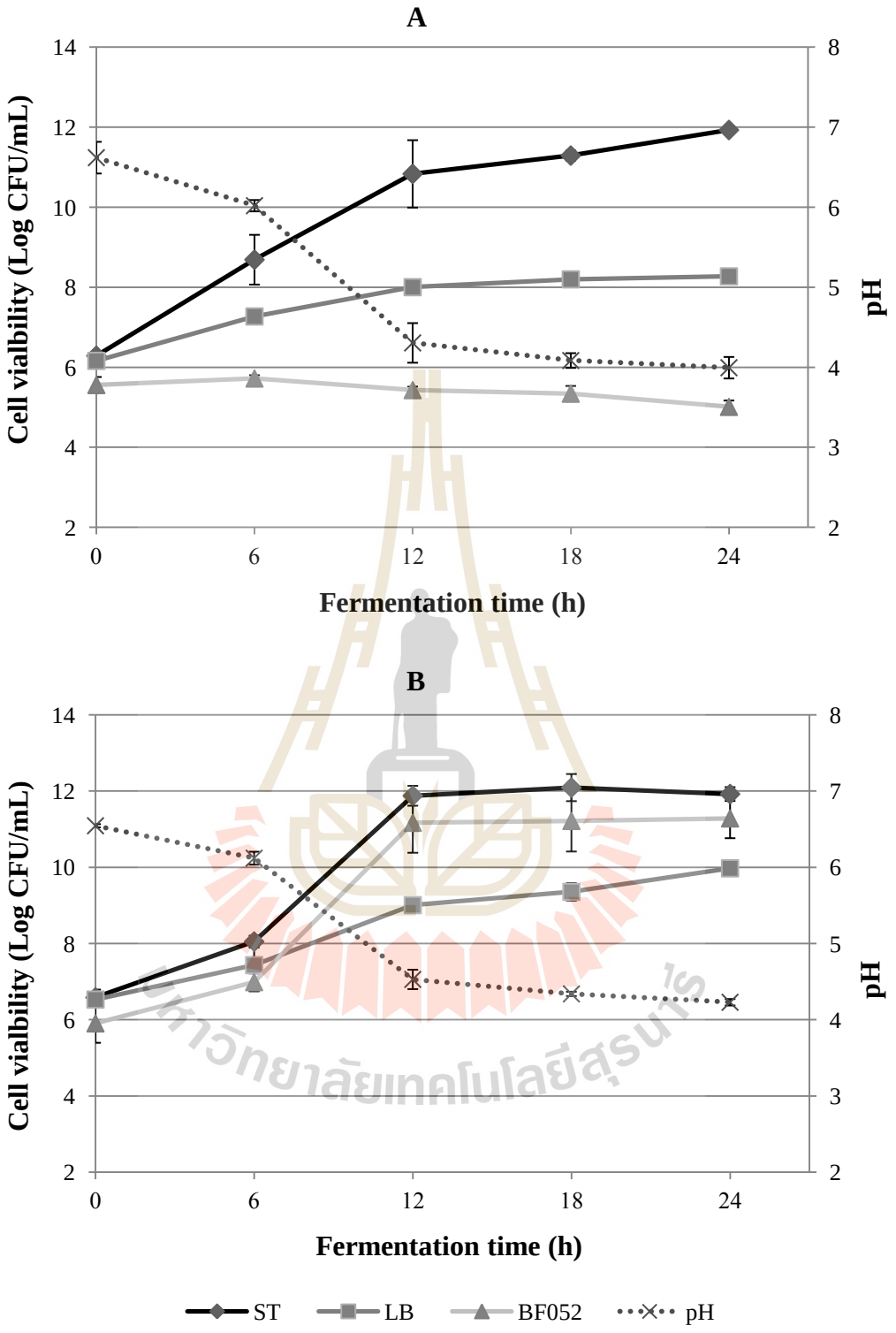


Figure 4.6 Growth and pH profiles of the mixed cultures of *S. thermophilus*, *L. bulgaricus* and *B. animalis* BF052 in milk (A) and soymilk (B) during fermentation for 24 h.

During fermentation in milk and soymilk supplemented with *B. animalis* BF052, *S. thermophilus* dominated with counts to reach 8 log CFU/mL since the first 6 h of fermentation time whereas *L. bulgaricus* grew slower and reach 8 log CFU/mL within 12 h (Figure 4.6). In comparison with milk, approximately 1 log higher numbers of *L. bulgaricus* was observed in soymilk after fermentation for 12 h. The growth patterns of the yogurt starters in both types of products were in line with Farnworth et al. (2007), which show that the *S. thermophilus* initiate the fermentation and that *L. bulgaricus* contribute to acidification later. Farnworth et al. (2007) also exhibited that 12 h was required to reach a pH value of 4.3 in both milk and soymilk, but the pH declined faster in the soy beverage than in the cows' milk. However, the culture mixtures in the current study needed different fermentation time to reach pH of 4.5, approximately 8 and 12 h for milk and soymilk, respectively. This suggested a greater rate of production of organic acids in the fermented milk than soymilk. The analysis of organic acids production during fermentation of milk indicated that significant increases ($p \leq 0.05$) in lactic acid correlated with the reduction in the lactose content (Table 5.4).

Table 4.4 Metabolic profiles of the strains during fermentation of milk during 24 h.

Fermentation time (h)	BF052			ST+LB+BF052		
	Lactose (g/L)	Lactic acid (g/L)	Acetic acid (g/L)	Lactose (g/L)	Lactic acid (g/L)	Acetic acid (g/L)
6	58.05±0.24^{a}	0.23±0.02^{a}	0.08±0.08^{a}	57.00±0.49^{d}	0.92±0.0.03^{a}	0.07±0.00^{a}
12	56.96±0.12^{a}	0.35±0.02^{a}	0.07±0.08^{a}	46.72±0.46^{c}	8.81±0.08^{b}	0.07±0.01^{a}
18	57.47±1.11^{a}	0.20±0.11^{a}	0.03±0.06^{a}	42.98±0.19^{b}	11.89±0.08^{c}	0.03±0.02^{a}
24	57.20±0.65^{a}	0.23±0.01^{a}	0.09±0.10^{a}	41.71±0.09^{a}	13.05±0.12^{d}	0.06±0.04^{a}

- ST : *S. thermophillus*, LB: *L. bulgaricus*, BF052 : *B. animalis* BF052

- The equal superscript lowercase letters in the same column indicate no significant differences (p>0.05).

Champagne et al. (2009) revealed that milk mostly contains lactose, while soy beverages have a variety of options of carbohydrate such as sucrose, stachyose, raffinose, glucose and fructose. This limited the ability of bifidobacteria to grow in milk due to low efficiency to assimilate lactose (Zare et al., 2012). Although bifidobacteria do not have a significant role either in the acidification of milk, or in the formation of texture and/or flavor, they are thought to play a health promoting role to the consumer (Prasanna et al., 2014). It is generally known that the most important objective of probiotic delivery is that the probiotic strains should be viable approximately 10^6-10^7 CFU/mL at the time of consumption and/or at the expiry date. However, whether bifidobacteria can grow in milk, it seemed that they do not attain as high numbers to achieve sufficient numbers before terminating the fermentation. Tabasco et al. (2014) demonstrated that *Bifidobacterium lactis* BB-12 could grow on lactose and milk with high β-galactosidase activity and reached populations above 9 log CFU/mL after fermentation for 24 h, however it required a high inoculation size at 7 log CFU/mL. Therefore, it is quite common to incorporate probiotic bacteria together with conventional starters. Regardless of the fermentation ability, Prado et al., 2016 suggested that probiotic bacteria may be directly added to the finished product, which lead to a higher number of viable cells and thus preserve its functionality. In addition, due to the buffering capacity of milk, it could ensure the survival of probiotics during fermentation and storage (Mani-López et al., 2014).

In recent year, numerous studies have been carried out on the enriching milk with supplements to enhance the growth of probiotics (Marafon et al., 2011; Yonezawa et al., 2010; Zare et al., 2012). The addition of a more readily available carbohydrate compound or other additives to milk could selectively enhance probiotic

growth during fermentation. In addition, this might offer the possibility of improving the formulation of fermented milk from both a bacterial growth enhancement and a nutritional perspective. On the other hand, much interest has been focused in the probiotic stability and functionality during processing and storage than the fermentation ability (du Toit et al., 2013; Kailasapathy et al., 2008; Laliĉić-Petronijević et al., 2015; Odamaki et al., 2011; Shori, 2015; Vinderola et al., 2013). These efforts reinforce the need for robust bifidobacteria that are able to survive stressful environmental challenges not only during industrial processes and storage but also after consumption through GI tract stresses. It was previously reported that *B. animalis* BF052 exhibited resilience to the adverse conditions not only during food-manufacturing chain but also under GI environments before adherence to the intestinal epithelium to exert health-promoting benefits there. In addition, *B. animalis* BF052 was found to exhibit high survival rates during refrigerated storage in pasteurized milk and drinking yogurt also (Charnchai et al., 2016). Although *B. animalis* BF052 lacks of role in fermentation performance, this strain still perceives ability of certain live microbe with high stability and functionality. Therefore, the development of fermented milk supplemented with *B. animalis* BF052 still required deep insights into its performances. This represents a great challenge for both science and industry sectors.

4.4 Conclusion

The utilization of dairy and non-dairy foods as probiotics carrier is representing potential advantages and valuable alternatives for the food industry and consumers. The results obtained in the present study demonstrated that the fermented

soymilk prepared with conventional starters and *B. animalis* BF052 would be an excellent vehicle for live probiotic bifidobacteria with respect to the synergetic growth between strains, low amount of acetic acid in the product, high consistency of yogurt texture and positive sensory scores. This study presents relevant information on microbial, physicochemical and sensory properties of probiotic *B. animalis* BF052 in fermented soy yogurt which could provide preliminary guidelines for developing a new probiotic-containing product to the industry.

CHAPTER V

WHOLE GENOME SEQUENCING OF *BIFIDOBACTERIUM ANIMALIS* BF052

5.1 Introduction

Members of the genus *Bifidobacterium* represent one of the most dominant groups in the intestines of breast-fed infants and are frequently used as probiotic ingredients due to their perceived role in the maintenance of the intestinal microflora balance (Turroni et al., 2011). *B. animalis* subsp. lactis BF052 (BF052) is a probiotic strain that originates in the feces of healthy breast-fed Thai infants. It shows probiotic potential with a high acid and bile tolerance and a strong adhesive capability to human intestinal epithelial. Our previous work demonstrated that BF052 exhibited resilience to the adverse conditions not only of the food-manufacturing chain, including freeze-drying, but also of GI environments before adherence to the intestinal epithelium to exert its health-promoting benefits (Charnchai et al., 2016). To gain insights into its adaptive responses to industrial and/or nutritional environments, the complete genome sequence of BF052 was therefore determined. The understanding of the genetic basis of BF052 is crucial for the improvement of a technologically-robust bifidobacterial strain for potential use in commercial application.

5.2 Materials and methods

5.2.1 Genomic DNA preparation

To extract genomic DNA, BF052 was precultured in MRSc broth at 37°C for 18 h, then 1% of preculture was transferred to fresh MRS broth and was incubated for 18 h. The cell culture was then harvested by centrifugation at 4,000 rpm for 5 min and washed once with Tris-ethylenediaminetetraacetic acid (EDTA) buffer (TE buffer; 25 mM Tris-HCl pH 8.0 and 5 mM EDTA). The cell pellet was collected and resuspended with 200 µl of 20% of sucrose in TE buffer solution. A 20 µl of lyzozyme (2 mg/mL) and 10% sodium dodecyl sulfate (SDS; 100 µl) were added to the cell suspension, then mixed by inverting tube followed by incubation at 37 ^{o}C for 1 h. After incubation, 75 µl of 5 M NaCl was added. Then, 500 µl of phenol: chloroform: isoammyl alcohol (ratio: 25: 24: 1) was added, mixed back and forth and centrifuged at 10,000 rpm for 10 min. The aqueous phase was collected and reextracted with the same solution until white precipitate at the interphase was not observed. To precipitate DNA, 500 µl of isopropanol and 50 µl of 3 M potassium acetate were added. The DNA was collected by centrifugation at 10,000 rpm for 5 min at 4°C, washed once with 70% ethanol and air-dried. The genomic DNA was then resuspended in 25 µl of sterile deionized water. Finally, the sample was treated with 10 mg/mL of RNaseA at 65°C for 10 min and stored at -20°C. The genomic DNA of BF052 was checked for integrity and lack of degradation by agarose gel electrophoresis and the concentration was measured by spectrophotometer (Nanodrop 2000, Thermoscientific).

5.2.2 Whole genome sequencing of BF052

The whole genome of BF052 was sequenced using the Illumina HiSeq platform (Baseclear, The Netherlands). To prepare Illumina DNA paired-end library, the genomic DNA was fragmented and ligated with DNA adapters at both ends of the DNA fragments. A size-selection was performed to select insert fragments in the range of 250-350±50 bp on average. Then, PCR amplification was performed and the resultant library was checked on a bioanalyzer and quantified. The library was then sequenced on the Illumina Sequencer using the Illumina Casava pipeline version 1.8.3 and sequence data was provided as FASTQ format.

The Illumina FASTQ reads were later assembled using the *De novo* assembly option of the CLC Genomics Workbench version 6.5.1 (CLC bio, Denmark). The generated contigs in total length of 1.9 Mbp were further concentrated from 51 contigs into 15 scaffolds using the SSPACE Premium scaffolder version 2.3 (Boetzer et. al, 2011). The gapped regions within the scaffolds were partially closed in an automated manner using GapFiller version 1.10 (Boetzer and Pirovano, 2012). Remaining gaps in the sequences were closed through primer walking on the amplified products. The complete genome was annotated using the NCBI Prokaryotic Genomes Automatic Annotation Pipeline (PGAAP).

5.3 Results and discussion

5.3.1 Genome features of BF052

General characteristics of BF052 genome are summarized in Table 5.1. The complete genome sequence of BF052 was composed of one circular chromosome

of a 1,938,624 bp circular molecule with no plasmid, carrying 1,538 coding genes, 12 rRNA operons and 52 tRNAs.

Table 5.1 *B. animalis* subsp. lactis BF052 genome features.

Attribute	**Value**
Genome size (bp)	1,938,624
G + C content (%)	60.5%
Protein coding genes (CDSs)	1,538
rRNA (5S, 16S, 23S)	12
tRNA	52

Nucleotide sequence accession number. The complete genome sequences of BF052 was deposited in GenBank under accession number CP009045.

Bifidobacteria are currently represented by over 30 species, which have been isolated mainly from GI tract of various animals and humans. *B. animalis* subsp. lactis is the most common bifidobacteria utilized as a probiotic in commercial dairy products. Currently, the deposited strains and their origins are listed in Table 5.2. Of the currently recognized 29 *B. animalis* (NCBI database), just 17 strains have been entirely sequenced and another 12 strains whose genome sequences are still unfinished.

Table 5.2 Currently assigned species of *Bifidobacterium animalis*.

Strain	Size (Mp)	Gene	Protein	Source	Aceesion No.	Reference
B. animalis subsp. lactis DSM 10140	1,938,483	1,610	1,534	French Yogurt	CP001606	(Barrangou et al., 2009)
B. animalis subsp. lactis AD011	1,933,695	1,614	1,518	Infant faeces	CP001213	(Kim et al., 2011)
B. animalis subsp. lactis Bl-04	1,938,709	1,611	1,538	Adult faeces	CP001515	(Barrangou et al., 2009)
B. animalis subsp. lactis BB-12	1,942,198	1,614	1,528	Fermented milk	CP001853	(Garrigues et al., 2010)
B. animalis subsp. lactis V9	1,944,050	1,613	1,541	Child feces	CP001892	(Sun et al., 2010)
B. animalis subsp. lactis CNCM I-2494	1,943,113	1,614	1,539	Unidentified source	CP002915	(Chervaux et al., 2011)
B. animalis subsp. lactis BLC1	1,938,583	1,611	1,539	Unidentified source	CP003039	(Bottacini et al., 2011)
B. animalis subsp. animalis ATCC 25527	1,932,693	1,586	1,478	Unidentified source	CP002567	(Loquasto et al., 2011)
B. animalis subsp. lactis B420	1,938,595	1,613	1,538	Unidentified source	CP003497	(Stahl and Barrangou, 2012)
B. animalis subsp. lactis Bi-07	1,938,822	1,611	1,538	Unidentified source	CP003498	(Stahl and Barrangou, 2012)
B. animalis subsp. lactis Bl12	1,938,606	1,611	1,537	Unidentified source	CP004053	(Milani et al., 2013)
B. animalis subsp. lactis ATCC 27673	1,963,012	1,628	1,488	Unidentified source	CP003941	(Loquasto et al., 2013)
B. animalis strain RH	1,931,057	1,607	1,537	Centenarian feces	CP007755	(Liu et al., 2014)
B. animalis subsp. lactis KLDS2.0603	1,946,899	1,614	1,529	Adult feces	CP007522	(Zhu et al., 2016)
B. animalis strain A6	1,958,651	1,626	1,545	Centenarian feces	CP010433	(Sun et al., 2015)
B. animalis subsp. lactis BF052	1,938,624	1,611	1,538	Infant feces	CP009045	(Charnchai et al., 2016)
B. animalis subsp. animalis YL2	1,800,480	1,486	1,390	Unidentified source	CP015407	Unpublished

5.3.2 Bifidobacteria and genetics of the stress response

The incorporation of bifidobacteria in food preparations requires that the strains should survive throughout food manufacturing processes, especially during starter handling and storage. In addition, bifidobacteria have to survive passage through the upper part of the digestive tract, while being able to compete with resident intestinal flora, colonize the digestive tract and express specific functions at the target sites. Therefore, a fundamental understanding of adaptive responses to environmental stresses from genetic information may provide methodologies to improve viability and functionality during commercial preparation, storage and delivery of the probiotic organism.

5.3.2.1 Temperature tolerance

In the manufacturing processes, bifidobacteria are normally subjected to temperature stresses when they are utilized for probiotic applications. In order to resist aggravating environmental conditions, bifidobacteria, like other bacteria, are capable of synthesizing a particular set of proteins protecting the cell from damage caused by the accumulation of unfolded and/or misfolded proteins (De Dea Lindner et al., 2007; Ventura et al., 2004). All nine complete genome sequences of bifidobacteria carry several temperature stress-related gene analogs, including genes encoding the chaperone families Hsp100 (ClpBCX), Hsp70 (DnaK, GrpE, and DnaJ), and Hsp60 (GroEL/GroES complex), which play key roles in several post-translational events to prevent protein denaturation, aggregation and misfolding (Lee and O'Sullivan, 2010).

The screening of BF052 genome sequence revealed chaperone-encoding genes involved in the heat stress responses, GroEL (GU89_03340), GroES (GU89_00640, GU89_01820), GrpE (GU89_07860), DnaK (GU89_07865), and DnaJ (GU89_04255 and GU89_07855). In addition, genes encoding cold-shock proteins (Csp) (GU89_03335 and GU89_03365) were found in the BF052 genome. It was suggested that Csp may involve in cryoprotective response during the freeze-drying process (Wouters et al., 2001). The caseinolytic proteases (Clp) were also identified in BF052 (GU89_03385, GU89_05455, and GU89_05460). This gene might be pivotal for the survival of the strain under heat, cold, and osmotic stresses (Jin et al., 2012).

5.3.2.2 Oxygen tolerance

Exposure of *Bifidobacterium* to oxygen is inevitable during handling in food-manufacturing processes. Even though bifidobacteria are generally described as strict anaerobes, their oxygen sensitivities are variable. Among other species, *B. animalis* subsp. lactis was reported as the highest oxygen-tolerant strain (Lee and O'Sullivan, 2010). Anaerobic bacteria differ in their sensitivity to oxygen due to the different activities of enzymes to remove reactive oxygen species (ROS), such as NADH oxidase, NADH peroxidase, and superoxide dismutase (SOD). Although previous studies have shown that bifidobacteria utilize NADH oxidase/peroxidase-like systems for the protection of their cells from ROS (Shimamura et al., 1992), the complete genome sequences of bifidobacteria revealed a gene analog only for the NADH oxidase gene. The reaction of oxygen and NADH with NADH oxidase produces and results in accumulation of hydrogen peroxide

(H_2O_2), which has many destructive properties, including inhibition of the F6PPK enzyme. The absence of catalase activity indicated that bifidobacteria had other mechanisms in the prevention of the accumulation of H_2O_2 during exposure to oxygen. However, none of the genome sequences of bifidobacteria contain gene analogs for NADH peroxidase, suggesting that there may be an alternative mechanism (Lee and O'Sullivan, 2010). Interestingly, all of the genome sequences of bifidobacteria, except for that of *B. adolescentis* ATCC 15703, reveal peroxiredoxin family alkyl hydroperoxide reductase gene analogs, which have been shown to be involved in the reduction of H_2O_2 in *E. coli* and *Streptococcus mutans* (Poole et al., 2000; Seaver and Imlay; 2001). An alternative system for H_2O_2 reduction to H_2O is the reduction of thioredoxin by thioredoxin reductase and NADH. Thioredoxin systems are prevalent in nature and are proposed to have multiple functions, including protection from oxidative stress (Arnér and Holmgren, 2000).

The genome sequence of BF052 revealed genes responsible for the reduction of H_2O_2, such as thioredoxin reductase (GU89_04390 and GU89_08190) and peroxiredoxin (GU89_02325). It was reported that peroxiredoxin genes were generally found in the genus bifidobacteria, except for some species of *B. adolescentis*, *B. breve*, *B. bifidum*, and *B. longum* (Lee and O'Sullivan, 2010). In addition, the BF052 genome contained oxidative damage repair-related genes, including those for nucleoside triphosphate pyrophosphohydrolase (MutT) (GU89_01035, GU89_02925, and GU89_04420) and ribonucleotide reductase (GU89_01665). Other predicted protein-coding genes involved in the reduction of oxidative damage, namely alkyl hydroperoxide reductase (AhpC) (GU89_04385) and

peptide methionine sulfoxide reductase (GU89_05290), were also indentified in the BF052 genome (Xiao et al., 2011).

5.3.2.3 Acid and bile tolerance

Besides temperature and oxygen stresses, bifidobacterial starters are generally subjected to low pH environments not only in fermented products in which they are finally formulated but also in the stomach during GI transit. In addition, bifidobacteria produce large amounts of organic acids, mostly acetic acid and lactic acid, as end products of sugar metabolism. These bacteria must therefore have evolved a system in maintaining their cytoplasmic pH near neutral. Ventura et al. (2004) proposed that acids pass passively through the bifidobacterial membrane and are rapidly dissociated into their impermeable constituent protons and charged derivatives. The maintenance of cytoplasmic pH requires that these protons are being neutralized or expelled from the cytosol. In this respect, the proton-translocating ATPase (F_0F_1-ATPase) system is involved in acid tolerance by pumping protons out of the cell.

Analysis of BF052 genome was shown to contain F_0F_1-type ATP synthase system (GU89_07235-GU89_07270) including cystathionine β-lyase (GU89_02195), cystathionine γ-synthase (GU89_02865) and cystathionine β-synthase (GU89_02870) responsible for survival under acidic stresses (Jin et al., 2012). On the other hand, genome analysis of *B. dentium* Bd1, a strain isolated from dental caries, revealed another acid tolerance system, the glutamate-dependent acid resistance system 2, which encodes a glutamate decarboxylase (GadB) and a glutamate/gamma-aminobutyrate antiporter (GadC). This system was not presented in other

bifidobacterial genomes including in BF052 genome (Ventura et al., 2009). When *B. dentium* Bd1 was exposed to acidic conditions, the F_0F_1-ATPase was not upregulated, indicating that this strain may manage its acid tolerance by amino acid degradation (Cronin et al., 2011).

The mechanisms allowing intestinal bacteria to resist physiological bile concentrations remain poorly understood (Lee and O'Sullivan, 2010). However, the activity of bile-salt hydrolases (BSHs) has been proposed to confer protection to bile stress through bile salt deconjugation (Ruiz et al., 2013). A choloylglycine hydrolase, belonging BSHs family enzymes which involved in the adaptation of bifidobacteria to bile-containing environments, was found in the BF052 genome (GU89_04375). Price et al., 2006 pre-exposed *B. longum* NCIMB 702259 cultures to cholate. The result showed an increase in resistance to sodium glycocholate and to a number of structurally unrelated antimicrobial compounds. The *crt* gene of *B. longum* demonstrated to encode a cholate transport system which is responsible for the efflux of [^{14}C] cholate for the cell and confer resistance to bile salts, chloramphenicol and erythromycin. However, the *crt* gene was not identified in BF052 genome. In addition, Sánchez et al. (2005) studied the effect of bile salt on protein expression patterns of *B. longum* NCIMB 8809. The result revealed that 34 different proteins were induced after both a minor (0.6 g/L) and a major (1.2 g/L) exposure to bile. These included general stress response chaperones, proteins involved in transcription and translation and the metabolism of amino acids and nucleotides, and several enzymes of glycolysis and pyruvate catabolism. The results suggested that bile salts, to which bifidobacteria are naturally exposed, induce a complex

physiological response rather than a single event in which proteins from many different functional categories take part.

5.3.3 Bifidobacteria and prebiotic properties

Many of the sugars that escape digestion by the host's enzymes are good carbon and energy sources for the components of the microbiota. These include fructo-oligosaccharides (FOS), galacto-oligosaccharides (GOS), gluco-oligosaccharides, xylo-oligosaccharides, inulin, starch, arabinoxylan and arabinogalactan, lactulose and raffinose. The indigestibility of these sugars reflects the paucity of human enzymes required for their degradation. However, such carbon sources, be designated as prebiotics, may specifically affect the host by selectively stimulating the growth and/or activity of beneficial bacteria or a limited number of pathogenic bacteria in the colon, thus improving host health. In this regard, bifidobacteria active in the lower parts of the colon probably derive their localized ecological success from their capacity to metabolize these non-digestible oligosaccharides (Turroni et al., 2011; Ventura et al., 2007). Therefore, an understanding of the metabolic activities through genomic data, in particular capability to utilize wide range of complex oligosaccharides, can reveal ways to enhance *in vivo* growth advantages, which aim to improve the health benefits to the host.

5.3.3.1 Fructo-oligosaccharides (FOS)

FOS represents the most widely used commercial prebiotic which is generally found in fruits and vegetables (such as onion and chicory),

constituting a varying number of fructose moieties connected by β-2,1 linkages ([β-D-Fru*f*-(2→1)]n-D-Fru*f*). Bifidobacterial fructofuranosidases are intracellular glycosyl hydrolases involved in the hydrolysis of the β-2,1 glycosydic bond between glucose-fructose, and/or fructose-fructose moieties present in fructooligosaccharides (Pokusaeva et al., 2011; Ventura et al., 2007). Analysis of *Bifidobacterium* genome revealed the presence of gene encoding β-fructofuranosidase in *B. adolescentis*, *B. breve*, *B. longum* biotype infantis, and *B. animalis* subsp. lactis (Van den Broek et al., 2008) including in *B. animalis* subsp. lactis BF052 (GU89_06305).

5.3.3.2 β-Galacto-oligosaccharides

To grow on milk or milk-derived substrates including lactose, lactose-derived GOS, β-galactosidases are essential for bifidobacteria to utilize such carbon sources that contain β-galactosidic linkages. Besides hydrolytic degradation activity, this enzyme also represents as the best studied group of bifidobacterial glycosyl hydrolases with transglycosylic activity that can be used for the synthesis of prebiotic substances, such as transgalacto-oligosaccharides (TOS), from lactose (Pokusaeva et al., 2011; Van den Broek et al., 2008). It was reported that the growth patterns of *B. bifidum* NCIMB41171 showed a preference towards lactose and GOS rather than simple carbohydrates (such as glucose and galactose). This strain was found to express four β-galactosidases (BbgI, BbgII, BbgIII and BbgIV) with apparently different hydrolytic and transglycosylic activities to hydrolyze different substrates, including β-D-(1→6) galactobiose, β-D-(1→4) galactobiose, β-D-(1→4) galactosyl-lactose and N-acetyllactosamine (Goulas et al., 2009). The genome sequence of BF052 revealed genes with respect to β-galactosidases (GU89_00270,

GU89_01365, GU89_02430, GU89_02460 and GU89_02470). Table 5.3 compares the β-galactosidase amino acid sequences of *B. bifidum* NCIMB 41171 with BF052 and the other strains. Alignment scores of the amino acid sequences of the identified β-galactosidases with other known homologous proteins showed that BbgII are well conserved among candidate bifidobacterial species with > 61.5% sequence identity whereas BbgIII are more distantly among species.

Table 5.3 Comparison of the β-galactosidase amino acid sequences of *B. bifidum* NCIMB 41171 with other β-galactosidases deposited in the NCBI database.

Enzyme source	Amino acid No.	% Consensus position	% Identity position	Accession No.
B. bifidum NCIMB 41171 **(BbgI)**	1,291	-	-	ABE27151
B. bifidum ATCC 29521	1,292	99.3	99.0	BAQ97366
B. animalis subsp. lactis BB-12	1,159	54.9	44.5	ADC85295
B. animalis subsp. lactis BF052	1,152	55.2	44.8	AJD88036
B. bifidum NCIMB 41171 (**BbgII)**	689	-	-	ABP87597
B. bifidum ATCC 29521	693	99.4	99.4	BAQ98551
B. animalis subsp. lactis BB-12	695	74.9	61.5	ADC85511
B. animalis subsp. lactis BF052	695	74.9	61.5	AJD88238
B. bifidum NCIMB 41171 **(BbgIII**)	1,935	-	-	ABE27152
B. bifidum ATCC 29521	1,935	99.2	99.0	BAQ97681
B. animalis subsp. lactis BB-12	695	6.5	2.9	ADC85066
B. animalis subsp. lactis BF052	701	9.3	4.8	AJD87828
B. bifidum NCIMB 41171 **(BbgIV**)	1,052	-	-	ABE00939
B. bifidum ATCC 29521	726	21.7	13.2	BAQ97379
B. animalis subsp. lactis BB-12	849	53.7	45.5	ADC85504
B. animalis subsp. lactis BF052	1,067	65.2	55.0	AJD88231

5.3.3.3 α-Galacto-oligosaccharides

Certain bifidobacteria, including BF052 strain, have been shown to grow on soymilk-derived α-galacto-oligosaccharides, such as the trisaccharide raffinose [α-D-Gal*p*-(1→6)-β-D-Glc*p*-(1→2)-β-D-Fru*f*], the tetrasaccharide stachyose [α-D-Gal*p*-(1→6)-α-D-Gal*p*-(1→6)-α-D-Glc*p*-(1→2)-β-D-Fru*f*], and the disaccharide melibiose [α-D-Gal*p*-(1→6)-α-D-Glc*p*]. To metabolize such α-galacto-oligosaccharides, bifidobacteria require α-galactosidase enzyme activity which has been identified and characterized in five bifidobacterial strains; *B. bifidum* JCM 1254 (Wakinaka et al., 2013), *B. adolescentis* DSM 20083 (Van Laere et al., 1999), *B. bifidum* NCIMB 41171 (Goulas et al., 2009), *B. breve* 203 (Zhao et al., 2008) and *B. longum* subsp. longum 105-A (Hirayama et al., 2012). Analysis of sequenced bifidobacterial genomes suggests that the majority of bifidobacterial strains encode at least one copy of an α-galactosidase-encoding gene. The BF052 genome was shown to contain three α-galactosidase-encoding genes (GU89_07770, GU89_08055 and GU89_08080). A phylogenetic analysis using full-length amino acid sequences of predicted α-galactosidase was constructed using the maximum likelihood method implemented in the PhyML program (v3.1/3.0 aLRT) (Figure 5.1). The α-galactosidase amino acid sequences of BF052 were compared with other sequences available in NCBI database including *B. animalis* subsp. lactis DSM 10140 (Balat_1537, Balat_1596 and Balat_1601), *B. adolescentis* ATCC 15703 (BAD_1525, BAD_1528 and BAD_1576), *B. bifidum* NCIMB 41171 (BBNG_RS0108615) and *B. breve* DSM 20213 (BBBR_1868). The results indicated that each predicted α-galactosidase of BF052 showed high similarity to other known bifidobacterial α-galactosidases, especially between the same species. However, the

high amino acid similarity does not necessarily indicate common structural and physicochemical properties of the enzymes (Goulas et al., 2009). Existence of an α-galactosidase in *Bifidobacterium* indicates the ability of the bacterium to utilize soybean oligosaccharides in the human GI tract and hence contribute towards its competitive advantage over other bacteria.

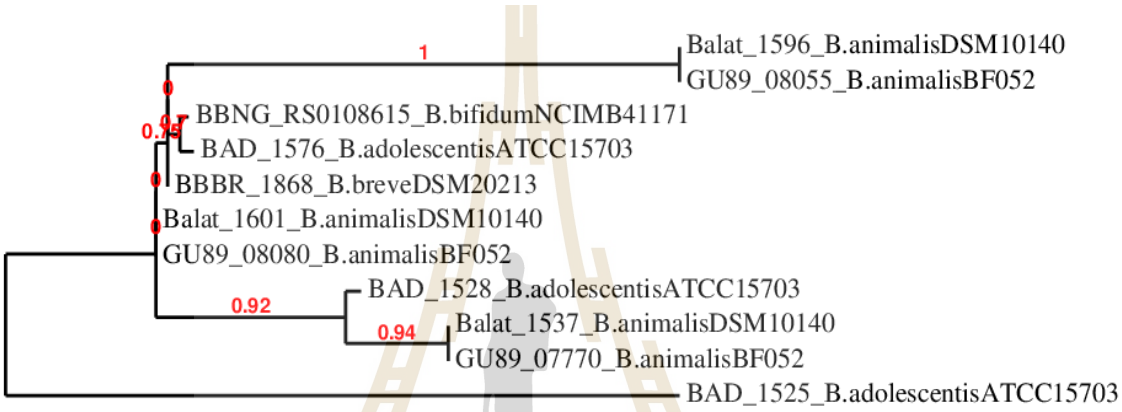


Figure 5.1 Phylogenetic tree of amino acid sequences of selected α-galactosidases. Bar scales indicate phylogenetic distances.

5.3.3.4 Arabinan, arabinogalactan, and arabinoxylan

Bifidobacteria have the capacity to ferment arabinofuranosyl-containing oligosaccharides derived from plant cell wall polysaccharides, such as arabinan, arabinogalactan and arabinoxylan, through the action of arabinoxylan arabinofuranohydrolases. Growth of *B. adolescentis* DSM 20083 on xylose and arabinoxylan-derived oligosaccharides was shown to induce the production of two different arabinofuranohydrolases. These enzymes were named arabinofurano-hydrolase-D3 (AXHd3; which hydrolyzed only C3-linked arabinose residues from double-substituted xylose residues) and AXHm23 (which released only arabinose

residues that were C2 or C3 linked to a single-substituted xylose residue). Both enzymes, together with a β-xylosidase, were able to degrade arabinoxylan-oligosaccharides completely (Van den Broek et al., 2005). The amino acid sequence of AXHd3 of *B. adolescentis* DSM 20083 (529 amino acids) was compared with arabino-furanohydrolase sequences found in the BF052 (519 amino acid; GU89_02650) as shown in Figure 5.2. The result revealed 52% identity with AXHd3.

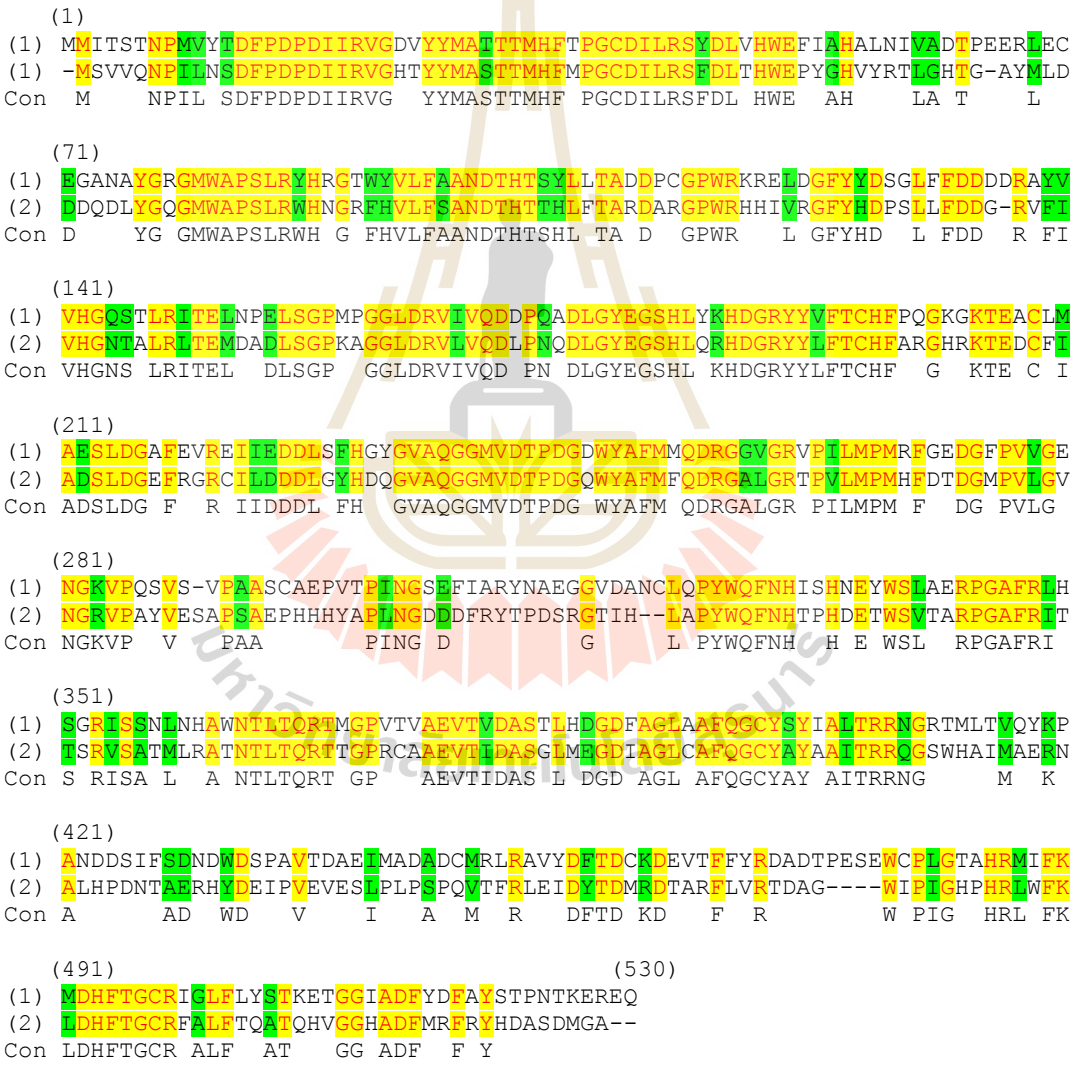


Figure 5.2 Amino acid sequence alignment of AXHd3 of *B. adolescentis* DSM 20083 (1) with arabino-furanohydrolase sequences found in the BF052 (2). Con: Consensus sequences.

5.3.3.4 Starch, pullulan and amylopectin

Starch is ubiquitous and is an easily accessible source of energy. It is composed exclusively of α-glucopyranose units that are linked to each other by α-1,4- or α-1,6-glucosidic bonds. The two high-molecular-weight components of starch are α-amylose (representing a 15 to 25% weight fraction of starch), which is a linear polymer composed exclusively of α-1,4-linked glucopyranose residues, and amylopectin (representing a 75 to 85% weight fraction of starch), which is also an α-1,4-linked glucopyranose polymer but in addition contains α-1,6-glycosidic linkages representing branch points occurring at every 17 to 26 residues. This enzymes hydrolyze α-1,4-glucosidic bonds, is capable of amylose degradation, yielding glucose, maltose, maltotriose, and other oligosaccharides. Genome of BF052 was found to contain gene encoding α-amylase (GU89_04830, GU89_07910, GU89_07945 and GU89_08045).

However, in the absence of a "debranching" enzyme capable of hydrolyzing α-1,6-glucosidic bonds, amylopectin degradation is incomplete. The α-1,6 bonds in amylopectin and pullulan are hydrolyzed by pullulanases, which are enzymes belonging to glycosyl hydrolase family 57 that are widely distributed in nature. Ryan et al. (2006) screened α-amylase and/or pullulanase activity from 42 bifidobacterial strains for their capacities to utilize starch, amylopectin, or pullulan. Eleven different bifidobacterial strains out of 42 tested, most of which belong to the *B. breve* species, indicating that the capacity to degrade these polymeric carbon sources may be a species-specific feature for *B. breve*. The screening of BF052 genome sequence revealed that predicted pullulanase-coding genes were also

indentified in the BF052 genome (GU89_04675, GU89_04680, GU89_04685 and GU89_07890).

5.4 Conclusion

The availability of genome sequences and molecular tools could provide a wealth of information on the important genus and which features are important for both probiotic uses and efficacy. The present report reveals relevant information of the genetic basis of a probiotic BF052 strain for adaptation to industrial stresses and nutritional environments. The understanding of the genomic basis of BF052 by interactive combination of genetic data and experimental approaches is crucial for the further development of a technologically- and functionally-robust bifidobacterial strain for potential use in commercially important applications.

CHAPTER VI

GENERAL CONCLUSIONS

The work presented in this dissertation has been accomplished the three objectives stated in the introduction:

1) To investigate the probiotic characteristics of isolated *Bifidobacterium* spp. and *Lactobacillus* spp. as a highly stable probiotic starter.

In the present study, only strain *B. animalis* BF052 satisfied the criteria as a potential probiotic based on its ability to survive through an *in vitro* model of GI conditions with the highest cell viability. This strain also possessed other considerable probiotic properties, including strong adhesion capability to Caco-2 cells (3.38 ± 0.15%), inhibitory activity against pathogens including *Salmonella typhimurium* and *Vibrio cholerae* and high survivability during refrigerated storage in a wide variety of the products. For technological aspects, the viability and functionality of the *B. animalis* BF052 strain was not affected by food processing chain, especially its resistance in the simulated GI conditions and its adherence ability to Caco-2 cells. These result indicated that *B. animalis* BF052 exhibited resilience to adverse conditions not only during industrial processes, including freeze-drying process, storage of freeze-dried powders, and incorporation of freeze-dried cells in food matrix, but also under GI environments before adherence to the intestinal epithelium to exert health-promoting benefits. Therefore, *B. animalis* BF052 studied in this research could be a potential probiotic candidate for beneficial use as an effective probiotic starter in food applications.

2) To develop soymilk as a delivery medium for the live probiotic *B. animalis* BF052.

The results obtained in the present study demonstrated that fermented soymilk prepared with conventional starters and *B. animalis* BF052 would be an excellent vehicle for live probiotic bifidobacteria with respect to the synergetic growth between strains, short fermentation time, low amount of acetic acid in the product, high consistency of yogurt texture and positive sensory scores. Therefore, soymilk supplemented with *B. animalis* BF052 could offer not only a means of enhancing beneficial health properties but also a possibility for improving flavor and texture of soymilk.

3) To reveal genetic features of the probiotic *B. animalis* BF052 from genomic sequence. Genes involved in adaptive responses to industrial and/or environmental stresses and in utilization of specific carbohydrates were indentified in *B. animalis* BF052 genome. The genomic data was translated into biologically relevant information which aimed to predict molecular mechanisms relevant to adaptive responses and health benefits. The understanding of the genetic basis of *B. animalis* BF052 by interactive combination of bioinformatics and experimental approaches is crucial for further improvement of a technologically- and functionally-robust bifidobacterial strain for potential use in commercial application.

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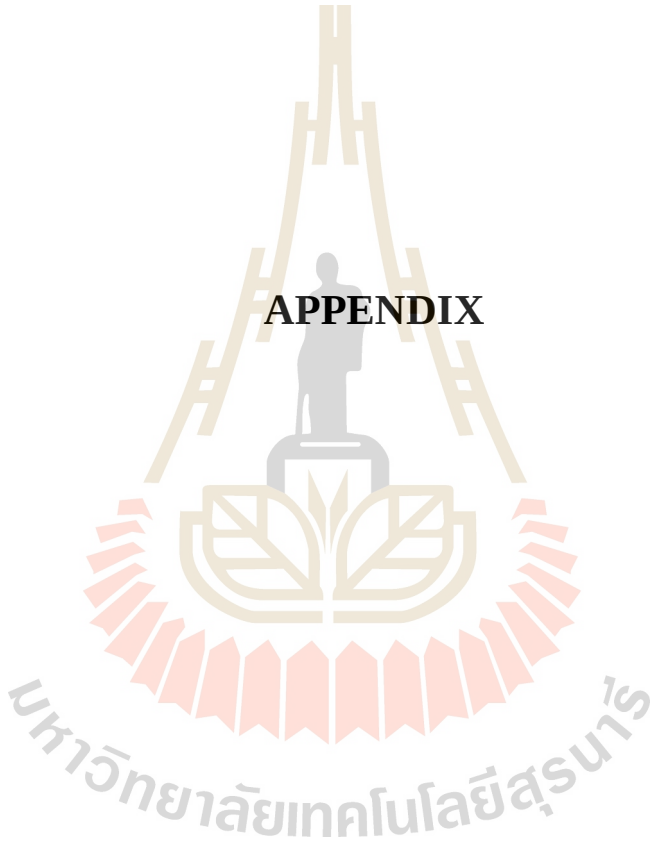
APPENDIX



APPENDIX

PLOS | ONE

RESEARCH ARTICLE

Effects of the Food Manufacturing Chain on the Viability and Functionality of *Bifidobacterium animalis* through Simulated Gastrointestinal Conditions

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Abstract

The viability and functionality of probiotics may be influenced by industrial production processes resulting in a decrease in probiotic efficiency that benefit the health of humans. This study aimed to investigate the probiotic characteristics of *Bifidobacterium* strains isolated from fecal samples of healthy Thai infants. In the present work, three local strains (BF014, BF052, and BH053) belonging to *Bifidobacterium animalis* showed a great resistance against conditions simulating the gastrointestinal tract. Among these, *B. animalis* BF052 possessed considerable probiotic properties, including high acid and bile tolerance, strong adhesion capability to Caco-2 cells, and inhibitory activity against pathogens including *Salmonella typhimurium* and *Vibrio cholerae*. This strain also exhibited a high survival rate compared to commercial strains during storage in a wide variety of products, including pasteurized milk, soy milk, drinking yogurt, and orange juice. The impact of food processing processes as well as the freeze-drying process, storage of freeze-dried powders, and incorporation of freeze-dried cells in food matrix on probiotic properties was also determined. The stability of the probiotic properties of the BF052 strain was not affected by food processing chain, especially its resistance in the simulated gastrointestinal conditions and its adherence ability to Caco-2 cells. It indicates that it satisfies the criteria as a potential probiotic and may be used as an effective probiotic starter in food applications.

Introduction

The consumption of health-promoting foods has developed in recent years together with an increasing variety of products conferring specific health benefits. In this regard, probiotic-

containing foods are highlighted as attractive products due to possess health promotion effects [1,2]. However, before probiotics can be of benefit to the health of humans, they must first be able to survive in sufficient numbers the manufacturing processes and storage as freeze-dried cultures, and also in the food products into which they are finally formulated. In addition, they should also possess the ability to survive the gastrointestinal (GI) tract and retain their functionality to be effective in the host [3,4]. Consequently, probiotic strains selected for commercial applications must retain the characteristics for which they were originally selected [5]. Recently, du Toit et al. [6] demonstrated that the same probiotic strain presented different characteristics depending on the manufacturing and processing conditions.

The manufacture of bifidobacterial cells at the recommended level of 10^6–10^7 CFU/ml of product represents a major technological challenge due to several factors that affect the viability of bifidobacteria during manufacturing and storage, such as the presence of oxygen, temperature, pH, and osmotic changes [2,7]. Production technology, in particular freeze-drying, manufactures powders containing a high number of viable cell cultures. Moreover, bifidobacterial cultures need to be able to survive during the shelf-life of the products [8]. The viability and stability of probiotics are challenges for industrial producers, and new technology has been developed to obtain highly stable probiotic starters with stable functionality [2,3,9]. A rigorous effort in strain selection and characterization is regarded as a prerequisite in this process [10]. This reinforces the need for robust bifidobacteria that are able to survive stressful environmental challenges not only during industrial processes such as freeze-drying, manufacturing, and storage but also after consumption through the GI tract stresses, until their adherence to the intestinal epithelium to exert health-promoting effects there [4]. Therefore, to guarantee a functional and effective probiotic strain with predictable health benefits, its viability and functionality throughout the food manufacturing processes and GI stress barriers must be investigated to ensure that health-promoting properties are maintained.

The aim of the present work was to investigate the probiotic characteristics of *Bifidobacterium* spp. previously isolated from fecal samples of healthy Thai infants as a highly stable probiotic starter. The candidate strains were initially screened on the basis of acid and simulated gastric tolerance and were further screened for functional properties, such as antimicrobial activity and adhesion ability. In addition, the study investigated the viability of the probiotic strains during storage in different food matrices. The authors consider this study to be a pioneer work in the evaluation of the impact of the production process chain on the selected probiotic's survival and resistance to GI stress, and its adhesion ability to Caco-2 cells.This part of the study was to ensure that the strain would still provide probiotic effects after consumption.

Materials and Methods

Microorganisms and culture conditions

Thirty bifidobacterial strains identified as *Bifidobacterium animalis* were selected from Suranaree University of Technology (SUT) culture collection, Thailand. These strains were previously isolated from fecal samples of healthy Thai infants. The widely used *Bifidobacterium animalis* subsp. lactis, strain BB-12 (Bb12) (Chr. Hansen, Denmark) was included in the experiments for comparison purposes. All strains were grown anaerobically at 37°C in DeMan, Rogasa and Sharpe (MRS; Oxoid Ltd., UK) broth supplemented with 0.05% L-cysteine hydrochloride (MRSc) and maintained in MRSc broth containing 20% (v/v) sterile glycerol and stored at -80°C.

The indicator organisms used for antimicrobial activity included *Escherichai coli* TISTR 780, *Staphylococcus aureus* TISTR 1466, *Pseudomonas aeruginosa* TISTR 781, *Bacillus cereus* TISTR 687, *Samonella typhimurium* TISTR 292, *Vibrio cholerae* O139, and *Candida albicans*

TISTR 718 which were supplied from the culture collection of the Laboratory of Microbiology, Institute of Science, Suranaree University of Technology, Thailand. All strains were cultured on Brain-Heart Infusion agar (BHI; Conda-Pronadisa, Spain) at 37°C for 16 h.

Resistance under conditions simulating the human gastrointestinal tract

The resistance of the examined strains under conditions simulating the GI tract was tested as previously described [11]. The tolerance was initially screened through low pH and simulated gastric juice. Briefly, bacterial cells from overnight (18 h) cultures were harvested (4,000 rpm, 10 min, 4°C) and washed twice with phosphate buffered saline (PBS; 0.8% NaCl, 0.2% KCl, 0.144% Na_2HPO_4, 0.024% KH_2PO_4, pH 7.2) supplemented with 0.05% L-cysteine hydrochloride (Merck, Germany) (PBSc), before being re-suspended in PBSc solution and adjusted to pH solutions of 2 and 3. For resistance to simulated gastric juice, bacterial cells were harvested and washed as described above. The bacterial suspension was then re-suspended in PBSc solution containing 0.3% (w/v) pepsin (Sigma-Aldrich, USA) and adjusted to pH solutions of 2 and 3. Resistance was assessed in terms of viable colony counts on MRSc agar after incubation of bacterial suspensions at 37°C for 0 and 3 h, reflecting the time spent by food in the stomach.

For resistance to small intestine conditions, bacterial cells as prepared above were re-suspended in PBSc solution containing 0.1% (w/v) pancreatin (Sigma-Aldrich, USA) and pH 8. The ability of the isolates to grow in the presence of bile was determined by adding cell suspensions to MRSc broth supplemented with 0.3%, 0.5 and 1% (w/v) bile salt (Oxoid Ltd., UK) and pH 8. The viable colony counts were determined after incubation at 37°C for 0 and 4 h, reflecting the time spent by food in the small intestine.

In vitro adherence assay

An adherence ability of the bifidobacteria was examined *in vitro* using Caco-2, a colonic adenocarcinoma cell line that expresses the morphological and physiological characteristics of normal mature human enterocytes. An adhesion assay was conducted as previously indicated by Pennacchia et al. [12]. Caco-2 cells were routinely grown in Dulbecco's modified Eagle's minimal essential medium (DMEM; Gibco, USA) supplemented with 10% (v/v) heat inactivated fetal bovine serum, 1% (v/v) L-glutamine, 1% (v/v) non-essential amino acid solution, and 1% (v/v) penicillin/streptomycin solutions (Gibco, USA) at 37°C in 5% CO_2 and 95% air atmosphere. Before the adhesion assay, overnight cultures of bacterial strains were harvested by centrifugation at 4,000 rpm and 4°C for 10 min (Centrifuge 5810R, Eppendorf, Germany). An aliquot of culture suspensions was serially diluted 10-fold in PBSc to determine the viable population by plate counting on MRSc agar after 48 h of incubation at 37°C. Another aliquot was re-suspended in non-supplemented DMEM (pH 7.0). This bacterial suspension was used to inoculate the six-well tissue culture plates with a concentration of about 10^8 CFU/mL.

The monolayer Caco-2 cells in the six-well tissue culture plates were washed twice with PBS and 2 ml of non-supplemented DMEM was added to each well. The plate was incubated at 37°C for 1 h. After incubation, non-supplemented DMEM was removed from each well and replaced by 1 ml of the bacterial suspension, prepared as described above. After incubation at 37°C for 90 min, the wells were softly washed 3 times with PBS to remove non-adherent bacteria. The washed monolayer was treated with 1 ml of 0.05% Triton X-100 water solution for 10 min to lyse the Caco-2 cells. The number of viable adhering bacteria was determined by plating serial 10-fold dilutions of the mixture containing lysed Caco-2 cells and bacterial cells on MRSc agar after 48 h of incubation at 37°C. The adhesion ability of the strains on Caco-2 cells was calculated as a percentage of the viable bacteria according to their initial population.

Antibiotic susceptibility test

Antibiotic susceptibility patterns of the strains were investigated by the disk diffusion method. The tested antibiotic discs (Oxoid, England) included streptomycin (10μg), gentamicin (10μg), tetracycline (30μg), penicillin G (10μg), aztreonam (30μg), vancomycin (30μg), erythromycin (15μg), chloramphinicol (30μg), kanamycin (30μg), ampicilin (10μg), lincomycin (15μg), norfloxacin (10μg), and ofloxacin (5μg). Strains were grown in MRSc broth at 37°C for 24 h under anaerobic condition to obtain a density of 10^7 cfu/mL. The culture suspension was swabbed on MRSc agar. Antibiotic discs were placed aseptically on the inoculated plates and agar plates were incubated anaerobically for 24 h at 37°C. The diameters of the inhibition zones around the discs were measured (average of three readings) and the results were interpreted according to the Clinical and Laboratory Standards Institute (CLSI, 2014) as sensitive (S), intermediate (I), and resistant (R).

Antimicrobial Activity

The ability of the candidate strains to inhibit the growth of pathogenic microorganisms was determined using the agar-well diffusion assay [13]. An overnight culture of the indicator strains was applied to inoculate in BHI agar at 37°C. Fresh overnight bifidobacteria cultures were harvested by centrifugation (4,000 rpm, 10 min, 4°C). The supernatants were neutralized to pH 6.5 and the others left unadjusted followed by filter-sterilization through 0.22 μm membrane filter. Cell-free extracts of bifidobacteria samples (100 μL) were pipetted into drilled holes (7mm) of the agar. The plates were then incubated at 37°C and were examined after overnight incubation. Antimicrobial activity was recorded as growth-free inhibition zones (mm) around the well.

Stability of probiotics in commercial products during storage

All candidate bifidobacteria were propagated in MRSc broth overnight at 37°C followed by sub-culturing and incubating for a further 18 h. All cultures were harvested by centrifugation and the pellets were then washed twice in PBSc solution, pH 7.4. A 1% inoculum of each bifidobacterial culture was aseptically distributed into 100 mL portions of four commercial dairy and non-dairy products (pasteurized milk, drinking yogurt, soy milk, and orange juice) to obtain a final concentration of 10^7–10^8 CFU/mL. Cell counts and pH measurements were performed immediately after the addition and every three days until 15 days of storage at a refrigerated temperature.

Preservation of bifidobacteria by freeze-drying in different cryoprotectants

A fresh overnight culture of a selected probiotic strain, *B. animalis* BF052, was grown in MRSc broth at 37°C. A 1% inoculum was then subsequently transferred to fresh MRSc broth. At the early stationary phase of growth (18 h), cells were harvested by centrifugation and washed twice with PBSc solution, pH 7.4. The pellet was re-suspended in 10% (w/v) lactose, 10% (w/v) sucrose, 10% (w/v) skim milk, 10% (w/v) germinated brown rice (GBR), 10% (w/v) black sesame (BS), and commercial soy milk. Sterile de-ionized water was used as a control. Aliquots (1 ml) of each cell suspension in different cryoprotectants were transferred into sterilized vials and frozen at -80°C for 4 h. Then, the samples were immediately freeze-dried for 18 h in a freeze-dryer (Alpha 1–2, Christ, Germany).

After freeze-drying, the freeze-dried powders were re-hydrated with MRSc broth (1 ml) and the cell suspensions were allowed to stand for 10 min at room temperature, and subsequently

plated on MRSc agar. The number of viable cells before and after freeze-drying was determined at 37°C after incubation for 48 h. To select the most effective cryoprotectant, freeze-dried samples were kept at room and refrigerated temperatures. After storage for 1, 3, and 6 months, the viability of the freeze-dried cells was then determined by plating on MRSc agar after 48 h of incubation at 37°C.

Gastrointestinal transit tolerance of BF052

The study investigated the effects of the production process chain, freeze-drying, storage of freeze-dried powders, and incorporation of cells in food matrix on the stability of the probiotic properties of *B. animalis* BF052. The strain *B. animalis* BF052 was subjected to a process of freeze-drying. The skim milk (10%) was used as a cryoprotective agent and the freeze-dried powders were then stored for 1 month following incorporation into a whole pasteurized milk and kept at refrigerated temperatures for 2 weeks. The strains were then sequentially exposed to simulated GI conditions followed by the adherence assay. To mimic *in vivo* human GI transit, an *in vitro* model was conducted as previously described by Peres et al. [1] and Sousa et al. [2], with slight modifications. After incorporation of the freeze-dried cells in a whole pasteurized milk for 2 weeks, 1 ml of products were transferred to a 34 ml of sterile electrolyte solution (SES; 0.22 g L^{-1} $CaCl_2$, 6.2 g L^{-1} NaCl, 2.2 g L^{-1} KCl, 1.2 g L^{-1} $NaHCO_3$, w/v) adjusted to pH 6.2. To simulate *in vivo* saliva conditions, 5 mL of a sterile electrolyte solution containing lysozyme (final concentration of 0.01% w/v) was added to 35 mL of cell suspension and incubated at 37°C, 200 rpm for 2 min. Then, 3 mL of the electrolyte solution (pH 5.0) with 0.3% (w/v) pepsin was incorporated into the cell suspension to simulate the oesophagus-stomach environment. The pH curve in the stomach was reproduced by adding 1 N HCl to the cell suspension to pH 6.0, 5.0, 4.0 every 10 min and to pH 3.0, 2.0 every 30 min, at 37°C, 50 rpm respectively. After 90 min of incubation, the samples were then adjusted to pH 5.0 using 1 M $NaHCO_3$ and mixed with 4 mL of a sterile electrolyte solution (5 g/L NaCl, 0.6 g/L KCl and 0.3 g/L $CaCl_2$, w/v), containing 0.3% (w/v) bile salts and 0.1% (w/v) pancreatin (pH 8) and incubated for 30 min (37°C and 50 rpm) to simulate the intestinal environment at the duodenum step. Finally, the ileum step was brought about by an increase of pH to 6.5 and incubation for 90 min at 37°C and 50 rpm. After passing through the GI step, cells were then sequentially tested for adherence ability. Briefly, bacterial solution was centrifuged and the pellet was re-suspended in 2 ml of non-supplemented DMEM. The monolayer Caco-2 cells in the six-well tissue culture plates were washed twice with PBS and 2 ml of non-supplemented DMEM was added to each well. The plate was incubated at 37°C for 1 h. After incubation, the non-supplemented DMEM was removed from each well and replaced by 2 ml of the bacterial suspension. After incubation at 37°C for 90 min, the wells were washed 3 times with PBS to remove non-adherent bacteria. The washed monolayer was treated with 2 ml of 0.05% Triton X-100 water solution for 10 min to lyse the Caco-2 cells. The number of viable adhering bacteria was enumerated by plating on MRSc agar after 48 h of incubation at 37°C.

Statistical analysis

Data were analyzed using SPSS 16.0 software (SPSS Inc., Chicago, IL, USA). Statistical differences in multiple groups were determined by one-way ANOVA followed by multiple mean comparisons with Duncan's test. All numerical data were displayed as mean ± standard deviation and $p \leq 0.05$ was considered statistically significant.

Results and Discussion

Resistance of bifidobacteria under conditions simulating the gastrointestinal tract

An essential step towards the selection of potential probiotic candidates is to examine their resistance under GI stress environments [1,14]. Out of 30 strains, only BF014, BF049, BF052, and BH053, including the reference strain Bb12, showed a decrease in viable counts lower than 1 log cycle even after 3 h of exposure at pH 3 (Table 1). No significant differences ($p>0.05$) in viable cells were observed in BF052 and Bb12 after an exposition to solutions with or without pepsin at pH 3 compared with initial counts. Although BF014, BF052, and BH053 did not survive after exposure at pH 2 for 3 h, all of the three strains consistently tolerated the pepsin solutions at pH 2 after 3 h of incubation. These results indicated that the bifidobacterial isolates (except strain BF049) were able to tolerate simulated gastric juice at pH 2 in the presence of pepsin. This result is relevant to the work of Mättö et al. [15] that showed the addition of inhibitors of pepsin and proton translocating enzyme significantly decreased the survival rate of *B. animalis* subsp. *lactis* at pH 2. Therefore, it was likely that pepsin was able to protect the cells during exposure to low pH by maintenance of the pH homeostatis and support of the role of H^+-ATPase. However, the loss of viability in BF049 after exposure to simulated gastric juice may indicate that the resistance to enzymatic barriers was strain-specific.

The strains resistant to stomach conditions were further tested for their ability to tolerate small intestinal conditions. All of the candidate strains were resistant to bile salts and pancreatic solutions at pH 8 by decreasing their viability approximately 1 log unit after 4 h exposure, as shown in Table 2. In general, the relevant physiological concentrations of human bile range from 0.3% to 0.5%. However, it was reported that bile salts were critical to bacterial cells since they disorganized the structure of the cell membrane [16]. However, all tested strains in this study retained their viability with small reductions at high concentrations of bile ranges from 0.3% to 1%.

In this study, three strains, BF014, BF052, and BH053, showed satisfactory probiotic properties for preliminary screening under conditions simulating the GI tract, suggesting that they may survive through the human GI transit. All of these three strains were therefore selected for the study of other probiotic properties.

Caco-2 cell adhesion

Adhesion of probiotic strains to human intestinal mucosa is regarded as a prerequisite characteristic for potential probiotic microorganisms. The adhesion ability to Caco-2 cells was

Table 1. Cell viability of probiotic strains after 3 h of exposure to low pH conditions and simulated gastric juice.

Strain	Initial count	low pH conditions		Resistance to gastric juice with 0.3% (w/v) pepsin	
		pH 2	pH 3	pH 2	pH 3
Bb12	$6.71 \pm 0.02^{\varphi,a}$	-[γ]	6.61 ± 0.05^{a}	6.21 ± 0.04^{b}	6.65 ± 0.01^{a}
BF014	7.14 ± 0.11^{a}	-	6.86 ± 0.25^{ab}	6.46 ± 0.05^{b}	7.08 ± 0.12^{a}
BF049	7.13 ± 0.03^{a}	-	6.93 ± 0.02^{b}	-	1.61 ± 0.02^{c}
BF052	7.31 ± 0.06^{a}	-	7.25 ± 0.07^{a}	6.99 ± 0.02^{b}	7.26 ± 0.05^{a}
BH053	7.79 ± 0.06^{a}	-	7.34 ± 0.18^{b}	7.19 ± 0.03^{b}	7.65 ± 0.03^{a}

[φ] Each value represents the mean value (log CFU/mL) ± stand deviation (SD) from three trials. The equal superscript lowercase letters in the same row indicate no significant differences ($p>0.05$)

[γ] No growth.

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Table 2. Cell viability of probiotic strains after 4 h of exposure to bile salt and pancreatin.

Strain	Initial count	Bile salt (pH 8)			Pancreatin (pH 8)
		0.3%	0.5%	1%	
Bb12	7.42 ± 0.14$^{\varphi,a}$	6.45± 0.08^{b}	6.43 ± 0.04^{b}	6.41 ± 0.09^{b}	6.42 ± 0.27^{b}
BF014	7.28 ± 0.12^{a}	6.51 ± 0.26 ^{b}	6.38 ± 0.03 ^{b}	6.43 ± 0.02 ^{b}	6.15 ± 0.14 ^{b}
BF049	7.27 ± 0.08^{a}	6.58 ± 0.03 ^{b}	6.58 ± 0.07 ^{b}	6.32 ± 0.09^{c}	5.10 ± 0.11^{d}
BF052	7.44± 0.06 ^{a}	6.80 ± 0.02 ^{b}	6.77 ± 0.01^{b}	6.73 ± 0.03 ^{b}	6.32 ± 0.15 ^{c}
BH053	7.43 ± 0.10^{a}	6.25 ± 0.18 ^{b}	6.23 ± 0.12^{b}	6.23 ± 0.15 ^{b}	6.31 ± 0.15 ^{b}

$^{\varphi}$ Each value represents the mean value (log CFU/mL) ± stand deviation (SD) from three trials. The equal superscript lowercase letters in the same row indicate no significant differences (p>0.05).

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evaluated and the result is presented in Table 3. The BF052 strain had a significantly higher adherence (3.38% ± 0.15) to Caco-2 cells comparable with the reference strain Bb12 (2.96% ± 0.12), whereas BF014 and BH053 expressed lower levels of adhesive abilities than those of BF052 and Bb12 strains. Sánchez et al. [17] revealed that adhesion values to the intestinal cell line HT29-MTX by *B. animalis* subsp. lactis IPLA4549 (2.96%±1.74) was slightly lower than Bb12 (3.08%±1.37). In addition, Laparra and Sanz [18] reported that Bb12 showed the highest adherence capability to Caco-2 cell and to human mucus (mucin type II) compared with other probiotic strains including *Lactobacillus rhamnosus* GG, *B. animalis* IATA-A2 and *B. bifidum* IATA-ES2. In this study, BF052 showed the highest percentage of adhesion compared to those candidate strains including the reference strain. As previously reported, the adhesion capability was not associated with species but as a characteristic of strain [19].

The adhesion of the microorganisms to the intestinal mucosa is an important feature involved in colonization and is related to the ability of the strains to interact with the host [20]. Probiotic bifidobacteria have several mechanisms that enable them to adhere to the intestinal epithelial cells. Their possible mechanisms may confer competition for substrates, direct antagonism by inhibitory substances, competitive exclusion of pathogenic bacteria, and potentially host-mediated effects, such as enhancing the function of the intestinal epithelial barrier by stimulation of the various signaling pathways and modulating immune responses [21–23]. As a result, high adhesive ability of bacteria to the cell lines may indicate that strains may contribute their beneficial effects to the host. However, investigations are still necessary to confirm their functionality in *in vivo* situations.

Antibiotic susceptibility assay

An important requirement for probiotic strains is that the isolated probiotics must be safe for human consumption. In this regard, antibiotic susceptibility profiles should be revealed and

Table 3. Adhesion ability of the isolates to Caco-2 cells.

Strain	% adhesion (mean±SD)*
Bb12	2.96 ± 0.12^{a}
BF014	2.57 ± 0.38^{b}
BF052	3.38 ± 0.15^{c}
BH053	2.72± 0.37^{ab}

*The equal superscript lowercase letters in the column indicate no significant differences between strains (p>0.05).

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Table 4. Antibiotic susceptibility profiles.

Type of antibiotics	Antibiotic susceptibility profiles			
	Bb12	BF014	BF052	BH053
Streptomycin (10μg)	R	R	R	R
Gentamicin (10μg)	R	R	R	R
Tetracycline (30μg)	S	S	S	S
Penicillin G (10μg)	S	S	S	S
Aztreonam (30μg)	R	R	R	R
Vancomycin (30μg)	S	S	S	S
Erythromycin (15μg)	S	S	S	S
Chloramphinicol (30μg)	S	S	S	S
Kanamycin (30μg)	R	R	R	R
Ampicilin (10μg)	S	S	S	S
Lincomycin (15μg)	S	S	S	S
Norfloxacin (10μg)	R	R	R	R
Ofloxacin (5μg)	R	R	R	R

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taken into account for safety [24]. Table 4 lists the antibiotic susceptibility patterns of the candidate strains and all candidates displayed similar phenotypic resistances comparable with the reference strain, Bb12. All tested strains were interpreted to be resistant towards aminoglycoside group (streptomycin, gentamycin, kanamycin), fluoroquinolone antibiotics (norfloxacin and ofloxacin), and β-lactam antibiotic (aztreonam, which is gram-negative spectrum). In contrast, all strains were sensitive to antibiotics belonging to a broad range of antibiotics related to different modes of action, such as β-lactam antibiotics (penicillin and ampicilin), broad-spectrum antibiotics (tetracycline and chloramphenicol), macrolide antibiotic (erythromycin), glycopeptide antibiotic (vancomycin), and lincosamide antibiotic (lincomycin). These antibiotic results indicated related patterns to previous reports [5,25,26]. From a safety point of view, it was proposed that a prospective probiotic should not carry transmissible antibiotic resistance genes, resulting in the corresponding genes not being transferred to the others including pathogens and commensal gut microbiota [27]. Probiotic strains with intrinsic antibiotic resistance may be thus useful for the restoration of the gut microbiota after antibiotic treatment [5]. Moreover, to the best of our knowledge, this is the first report in which all tested strains conferred resistance to norfloxacin and ofloxacin. Therefore, it is beneficial for patients suffering from urinary tract infection to restore the *Bifidobacterium* population after treatments involving norfloxacin and ofloxacin.

Antimicrobial Activity

For the antimicrobial assay, there was no observation of inhibition for any of the supernatants in which the pH was neutralized (results not shown). However, the non-neutralized culture supernatants of BF052 and BH053 strains showed inhibitory activities against *S. typhimurium* and *V. cholerae* as shown in Table 5. These results indicated that the most likely explanation was that the inhibition was due to organic acid production by the strains. Our results were in an agreement with previous works. Strompfová and Lauková [28] demonstrated that inhibition effects were not explained by bacteriocin action and were most probably due to the production of organic acids along with pH lowering effects during the growth in *Bifidobacterium*. Arboleya et al. [25] also reported that non-neutralized supernatants of breast-milk isolates (*B. longum* and *B. breve*) were able to inhibit *Salmonella enteric* and *Shigella sonnei*. Ibrahim and Bezkorovainy [29] demonstrated that no antibacterial substances were detected in the fermentation

Table 5. Inhibitory effects of non-neutralized bifidobacterial supernatants against pathogens.

Strains	Diameter (mm) of inhibition zones						
	E. coli	*S.aureus*	*P. aeruginosa*	*V. cholerae*	*B. cereus*	*S. typhimurium*	*C. albicans*
Bb12	-[a]	-	-	-	-	-	-
BF014	-	-	-	-	-	10	-
BF052	-	-	-	8	-	10	-
BH053	-	-	-	9	-	11	-

[a] No antagonistic activity was observed.

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broth of tested bifidobacteria. Only acetic and lactic acids were produced and could inhibit the pathogenic strain of *E. coli*. In addition, Fukuda et al. [30] proposed that the production of acetate by *B. longum* subsp. longum JCM 1217, *B. longum* subsp. infantis 157F, and *B. longum* subsp. longum NCC 2705 was able to protect mice against death induced by enterohaemorrhagic *Escherichia coli* O157:H7. However, Liu et al. [31] recently found a novel broad-spectrum bacteriocin called bifidocin A that is produced by *B. animalis* BB04. Therefore, it is likely that the antimicrobial activity of bifidobacteria may be implemented not only by the production of organic acids but also by the secretion of bacteriocin.

Storage stability of probiotics in commercial products

Many criteria have been suggested for the selection of probiotics. Besides the challenge to overcome the GI stresses, the ability of probiotics to survive in products during storage is also important. It was recommended that the level of probiotics in food products needed to be high, suggesting the minimum counts of live cells should be at least 10^6–10^7 CFU/mL before consumption [2, 32]. This requirement has a significant impact on the selection of potential probiotics with high stability in different food products.

In the present study, strains Bb12, BF014, BF052, and BH053 were incorporated into dairy (pasteurized milk and drinking yogurt) and non-dairy products (soy milk and orange juice) at refrigerated temperatures for 15 days. Fig 1 displays viable cells in refrigerated storage over 15 days. No significant differences ($p>0.05$) were observed in all the candidate strains in cultivable cell numbers during storage in pasteurized milk and soy milk during the 15 days. In drinking yogurt, a significant decrease ($p\leq0.05$) in cell viability was detected only in strain BH053 after storage for nine days (S1 Table). A major significant reduction ($p\leq0.05$) in cell counts ranging from 0.6 to 1.0 log cycles was observed in orange juice in all tested strains. These results are in agreement with those of Saarela et al. [33] and Vinderola et al. [34] who reported that the stability of bifidobacterial cells in the low pH of fruit juice was poorer than the fairly neutral pH of milk during refrigerated storage. Nualkaekul et al. [35] proposed that the presence of protein sources in food matrices may improve the survival of bifidobacteria during refrigerated storage. This was in accordance with the present study's findings that high amounts of proteins in drinking yogurt may have resulted in a higher rate of cell survival than in juices, although pH values of both products were slightly different. It was proposed that when probiotic cells were present in low pH environments, the requirement of energy consumption increased to maintain the intracellular pH, resulting in depression of ATP for crucial cellular functions and thereby causing cell death. In addition, exposure to oxygen under acidic conditions during refrigeration storage was most probably responsible for the reduction in probiotic counts [36, 37]. Among all the candidates, BF052 showed the highest survival rate during storage in all products, while the reduction rates of BH053 in terms of viable counts were significantly higher than those of other strains.

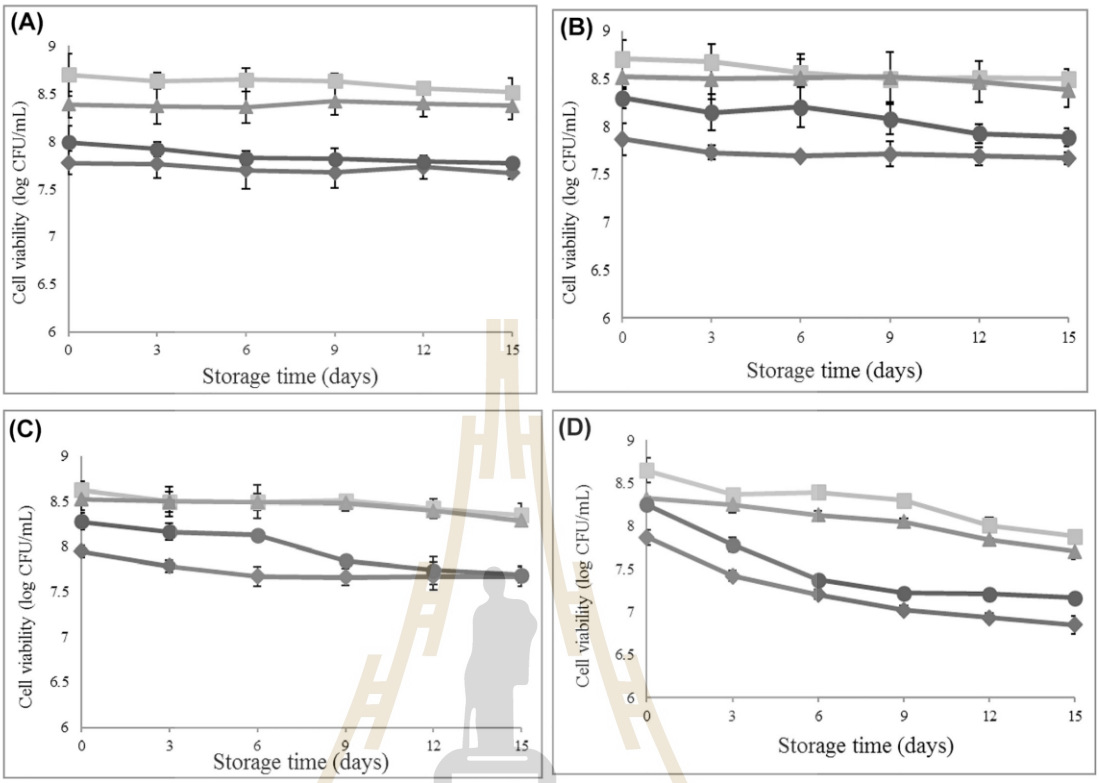

Fig 1. Cell viability of probiotics in refrigerated storage over 15 days in (a) pasteurized milk, (b) soy milk, (c) drinking yogurt and (d) orange juice. Symbols: Bb12 (◆), BF014 (■), BF052 (▲), and BF053 (●).

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Additionally, changes of pH values of BH053 slightly declined compared to those of other strains, especially in soy milk, whereas BF052 remained constant during the incubation period (data not shown). The reduction of the pH value of BF052 in only soy milk (within 0.17 pH-values) but no other products was observed. This result was in line with previous studies which showed the decrease of the pH levels in the soy beverage was faster than in milk [38, 39] suggesting a greater rate of organic acid production. It was also observed that soy milk containing oligosaccharides, such as raffinose and stachyose, may support the growth of bifidobacteria causing acid production and subsequent reduction of pH. However, post-acidification during storage is an undesirable property in probiotic-containing products. This process may have adverse effects on the taste or aroma of the product and may cause a loss in the viability of the probiotic strain [40].

The main purpose of the present study was to identify suitable probiotic strains for incorporation into food products. It was clearly observed that candidate strains belonging to the same species may present different characteristics even in food matrices. Among all candidates, BF052 was found to exhibit the highest survivability in a wide variety of the products,

suggesting that it may have been present in sufficient amounts throughout the entire shelf life of the product. In addition, BF052 possessed considerable probiotic properties including high acid and bile tolerance ability, strong adhesion capability, and good inhibitory activity against pathogens. This strain was thus selected as a promising probiotic strain that may have potential as probiotic starter.

Preservation of BF052 by freeze-drying in different cryoprotectants

In industrial applications, the use of probiotics as starter cultures is required to guarantee long-term delivery of stable cultures in terms of cell viability and functionality [41]. Freeze-drying is a well-documented technique used for the preservation of microorganism [42]. Moreover, the utilization of a suitable cryoprotective as a freeze-drying agent is an achievable attempt to improve cell viability during this process. In this study, sucrose, lactose, skim milk, GBR, BS, and soy milk were examined for their ability to protect the BF052 cells during freeze-drying. Table 6 shows the effect of cryoprotectants on the survivability of BF052 at different storage periods and temperatures.

Among all candidate cryoprotectants, only 10% skim milk showed no statistical difference ($p>0.05$) in protecting cells during freeze-drying. After storage, the survival rates of the BF052 freeze-dried cells were better at refrigerated temperatures than room temperature. It was reported that powdered *Bifidobacterium* preparations survived better in refrigerated storage than at room temperature [33]. In addition, there were no significant differences ($p<0.05$) in the viable cells using soy milk and BS after storage of freeze-dried powders for 1 month at refrigerated temperature and room temperature. However, after a month storage, the cell viability after 1 month storage in soy milk was higher than that of BS.

According to Carvalho et al. [43], distinct properties of the cryoprotectants resulted in different protection features. The protective ability of skim milk on freeze-dried cells may be explained by its capacity in the prevention of cellular injury, stabilization of the cell membrane constituents, and provision of a protective coating for the cells [44]. Vinderola et al. [33] also found that skim milk and lactose were effective in the protection of *B. animalis* subsp. lactis INL1 comparable with sucrose during freeze-drying and storage, including after exposure under the harsh conditions of simulated digestion. Besides skim milk, soy milk is especially interesting as an attractive cryoprotectant. It is likely that soy milk contains many substances

Table 6. Effects of cryoprotective agents on cell survival of BF052 during freeze-drying (FD) and storage.

Cryoprotectants	Cell viability (log CFU/mL ± SD)		Cell viability after storage in refrigerator (log CFU/mL ± SD)			Cell viability after storage at room temperature (log CFU/mL ± SD)		
	Before FD	After FD	1 month	3 months	6 months	1 month	3 months	6 months
DI water	9.32 ± 0.07^{a}	$8.60 \pm 0.2\text{-}^{b}$	8.42 ± 0.02^{b}	8.10 ± 0.10^{c}	7.79 ± 0.04^{d}	6.09 ± 0.08^{e}	-	-
10% Sucrose	9.32 ± 0.27^{a}	9.02 ± 0.01^{b}	8.73 ± 0.01^{c}	8.68 ± 0.01^{cd}	8.44 ± 0.02^{d}	6.26 ± 0.19^{e}	$-^{\pi}$	-
10% Lactose	9.34 ± 0.22^{a}	8.99 ± 0.10^{b}	8.75 ± 0.02^{bc}	8.76 ± 0.08^{bc}	8.65 ± 0.04^{d}	7.80 ± 0.03^{e}	6.02 ± 0.16^{f}	3.10 ± 0.04^{g}
10% Skim milk	9.21 ± 0.07^{a}	9.16 ± 0.02^{a}	9.16 ± 0.01^{a}	9.15 ± 0.01^{a}	9.12 ± 0.01^{a}	8.78 ± 0.10^{b}	7.42 ± 0.08^{c}	6.06 ± 0.03^{d}
10% Germinated brown rice	9.81 ± 0.32^{a}	9.41 ± 0.23^{ab}	9.19 ± 0.12^{bc}	8.90 ± 0.10^{c}	nd	7.61 ± 0.19^{d}	5.14 ± 0.13^{e}	nd
10% Black sesame	9.36 ± 0.03^{a}	8.48 ± 0.65^{b}	8.39 ± 0.06^{b}	8.31 ± 0.08^{b}	nd	7.76 ± 0.16^{b}	6.58 ± 0.15^{c}	nd
soy milk	9.20 ± 0.27^{a}	8.93 ± 0.10^{ab}	8.87 ± 0.09^{ab}	8.83 ± 0.09^{ab}	nd	8.77 ± 0.07^{ab}	7.33 ± 0.14^{c}	nd

- The equal superscript lowercase letters indicate no significant differences between cryoprotectant ($p>0.05$)

$^{\pi}$ No growth

nd Not determined.

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in protecting BF052 freeze-dried cells, such as protein, which is equivalent to that of milk, soybean-oligosaccharides, stachyose, and raffinose. Moreover, it was reported that the survival of *B. animalis* subsp. lactis 10140 during the freeze-drying process was enhanced by the presence and increment of probiotics [45]. In contrast to skim milk and soy milk, GBR and BS are composed of mostly polymeric sugars. They easily form glasses that often do not have suitable structures to be able to depress membrane phase transition resulting in failure to protect microbial cells during the freeze-drying process [46]. Zhao and Zhang [41] suggested that a good cryoprotection should protect the cells during the freezing process, be easily dried, and provide a good matrix to allow stability and ease of rehydration. During rehydration using MRS broth, GBR and BS were not perfectly rehydrated due to complex substances and thus affected the survival rate of freeze-dried cells.

Nowadays, the demand for non-dairy probiotic products has increased and the use of soy milk as a cryoprotectant during the freeze-dried process is an option to develop a fully non-dairy probiotic product. However, dairy products are still the main vehicles for the incorporation of probiotic cultures [5, 47]. In this study, skim milk was the most effective protective agent for BF052 cells during freeze-drying and storage, and was therefore selected for further study.

Gastrointestinal transit tolerance of BF052

This study aimed to examine the consistency of the probiotic properties of BF052 after the production process, including freeze-drying, storage, and incorporation of the strain into the products. After this process, BF052 was evaluated the tolerance ability through an *in vitro* model of the human GI tract. The strain was encountered the lysozyme-containing saliva in the mouth, pH gradient and gastric enzymes in the stomach, followed by the bile and pancreatic enzymes in the small intestine, and the adherence of the strain to human intestinal mucosa as a final step. Changes in cell viability by the end of each stage were examined.

Strain BF052 showed the ability to resist to the adverse conditions tested in every compartment as shown in Table 7. It exhibited a small susceptibility through each step, with different enzymatic- and pH-dependent barriers until gastric emptying at increasingly lower pH (reaching to pH 2.0). A significant reduction ($p \leq 0.05$) in cell survival occurred only at pH 2 in all processes. This strain was also resistant to the duodenum and ileum steps and retained its viability with a small reduction in viable counts. These results are consistent with those previously revealed from other *B. animalis* strains belonging to *B. animalis* Bb12 [2] and *B. animalis* Bo [48], which generally showed a great resistance throughout the whole processes of simulated digestion.

In addition, the impact of food manufacturing processes, such as freeze-drying, was also determined and compared with the direct adherence assay. The results showed that no significant differences ($P > 0.05$) in adhesion capability were detected among freeze-dried and non-freeze-dried cells. This result was in contrast to Du Toit et al. [6] who reported that freeze-drying of probiotics was found to have an adverse effect on adhesion capability. Osmotic shock, formation of intracellular ice, and re-crystallization during freeze-drying may damage the biological structures of the cell and probably affect the adhesion ability of probiotics. However, use of an appropriate cryoprotectant during freeze-drying may reduce such adverse changes resulting in the maintenance of the ability of this strain to exhibit probiotic behavior [9]. However, our experiments also demonstrated the effect of freeze-drying process on adhesion ability of probiotics after passage through the conditions of the GI tract. Based on our results, it was observed that the introduction of BF052 through the GI transit may enhance the adhesive ability to Caco-2 cells compared with those of non-challenged conditions. It may be explained that

Table 7. Cell viability (log CFU/mL ± SD) within dynamic *in vitro* model and adhesion capability of *B. animalis* BF052 from different processes.

Process	Initial count	Gastrointestinal compartment								% Adhesion
		Mouth	Oesophagus-stomach					Duodenum	Ileum	
		2 min	pH 6 10 min	pH 5 10 min	pH 4 10 min	pH 3 30 min	pH 2 30 min	pH 5 30 min	pH 6.5 90 min	
BF052-Caco2	8.13 ± 0.26	-	-	-	-	-	-	-	-	3.19% ± 0.11[A]
BF052-GI test-Caco2	8.47 ± 0.39 [a]	8.47 ± 0.40 [a]	8.48 ± 0.42 [a]	8.46 ± 0.32 [a]	8.43 ± 0.36 [a]	8.38 ± 0.41 [a]	7.21 ± 0.49 [b]	7.09 ± 0.44 [b]	6.99 ± 0.49 [b]	3.81% ± 0.32[A]
BF052-FD-Caco2	8.13 ± 0.18	-	-	-	-	-	-	-	-	3.08% ± 0.15[A]
BF052 -FD-GI test-Caco2	8.34± 0.21 [a]	8.23 ± 0.21 [a]	8.32 ± 0.17 [a]	8.29 ± 0.24 [a]	8.27 ± 0.21 [a]	8.19 ± 0.11 [a]	7.01 ± 0.20 [b]	6.85 ± 0.11 [b]	6.77 ± 0.18 [b]	3.45% ± 0.21[A]
BF052-FD -milk-GI test-Caco2*	8.53 ± 0.21 [a]	8.49 ± 0.20 [a]	8.50 ± 0.24 [a]	8.48 ± 0.23 [a]	8.43 ± 0.21 [a]	8.39 ± 0.27 [a]	7.45 ± 0.14 [b]	7.35 ± 0.20 [b]	7.30 ± 0.19 [b]	3.67% ± 0.50[A]

- The equal superscript lowercase letters in the same row indicate no significant differences between digestion steps ($p>0.05$)

- The equal superscript capital letter in the last column indicates no significant differences in adhesion percentage for each process ($p>0.05$)

*In this process, *B. animalis* BF052 were freeze-dried (FD) by using 10% skim milk as a cryoprotectant agent and then stored as freeze-dried powders for 1 month at refrigerated temperature following incorporation into a whole pasteurized milk and kept at refrigerated temperature for 2 weeks before exposure through GI (GI) transit followed by adherence assay (Caco2).

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either acid or bile adaptation appeared to affect the *in vitro* adhesion to the intestinal cell line. Also, it was reported that the induction of acid or bile resistance in bifidobacteria may improve cellular surface properties and thus enhance the adhesion ability that favors their potential functionality as probiotics [49,50,51].

Before delivering probiotic-containing products to consumers, probiotic bacteria should survive and retain their functionality not only during storage as freeze-dried cultures but also in the food products into which they are finally formulated [52]. Eventually, it would be beneficial that the strain would be supplemented into the whole pasteurized milk as one of the alternative means for delivering probiotics. In this study, after freeze-drying and the subsequent storage as freeze-dried powder for 1 month, BF052 were sequentially delivered in a whole pasteurized milk as a probiotic vehicle and stored at refrigerated temperatures for two weeks. The survival of the strain throughout the process of simulated digestion was then monitored. Interestingly, the whole production process did not affect the stability of the probiotic properties of BF052, especially the resistance of this strain through the GI transit, including adherence ability. BF052 still displayed a similar ability to withstand GI stresses and exhibited no significant variations ($P>0.05$) in adhesive ability to Caco-2 cells despite differences in cell preparations. Moreover, it was observed that carriers of probiotic bacteria were involved in affecting the viability and functionality of probiotics during storage and throughout the simulated GI system [53]. Kos et al. [54] studied the effect of whey protein concentrate (WPC) on the viability of *L. acidophilus* M92, and found that addition to WPC may protect the cells from the low pH of simulated gastric juice, and even higher concentrations of bile salts. In addition, Madureira et al [48] proposed that whey cheese matrices as a probiotic vehicle were shown to protect *L. casei*, *L. acidophilus*, and *B. animalis* during *in vitro* simulated digestion, compared with their performance in plain MRS medium. Saarela et al. [32] also reported that acid and bile tolerances were better in freeze-dried *B. animalis* subsp. lactis E2010 added to pasteurized milk compared with those in phosphate-buffered saline or juice held at 4°C over two weeks. Therefore, several factors may influence the ability of the probiotics to survive in the product and become active when entering the consumer's GI tract. In this regard, the interactions of probiotics with the food matrix or the starter culture, pH, acidity, temperature, and oxygen content of the product are also important.

Probiotic strains selected for commercial application in foods must retain the characteristics for which they were originally selected [5]. In this report, even though the strains encountered potentially stressful conditions throughout the manufacturing processes and biological barriers during the GI transit, BF052 still maintained its original characteristics. These included the characteristics of survival and tolerance during manufacture and after consumption, and during transit through the stomach and small intestine until adherence to the intestinal epithelium. Therefore, it is anticipated that BF052 retains its probiotic functionality and remains viable at levels necessary to provide health benefits to consumers. However, *in vivo* investigations are still necessary to fully validate its beneficial roles to the health of human hosts.

Conclusion

It is crucial to investigate interesting strain characteristics in terms of safety and functional aspects for probiotic potential. In this regard, resistance of strains against production, storage, and GI tract stresses is of prime importance. This research demonstrated that *B. animalis* BF052 displayed promising probiotic properties and exhibited resilience to adverse conditions not only during industrial processes but also under GI environments before adherence to the intestinal epithelium to exert health-promoting benefits there. Therefore, *B. animalis* BF052 studied in this research is a potential probiotic candidate for further development as an effective probiotic starter.

Supporting Information

S1 Table. Data for cell viability (log CFU/ml) in various products after 15 days storage.
(DOC)

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Author Contributions

Conceived and designed the experiments: KJ SSJ PC. Performed the experiments: PC SSJ. Analyzed the data: KJ SSJ PC CP. Contributed reagents/materials/analysis tools: KJ SSJ CP SK. Wrote the paper: KJ PC.

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BIOGRAPHY

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