Screening of Probiotic Bacteria Isolated from Infant Feces for Development of Synbiotic Product

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ABBREVIATIONS AND SYMBOLS

ADI	Arginine deiminase system
AU	Arbitrary unit
BATH	Bacterial adhesion to hydrocarbon
BSA	Bovine serum albumin
BSH	Bile salt hydrolase
CD	Cesarean delivery
CFCS	Cell-free culture supernatant
CFU	Colony forming unit
CRC	Colorectal cancer
CTAB	Cetyl trimethyl ammonium bromide
DGGE	Denaturing gradient gel electrophoresis
FISH	Fluorescent *in situ* hybridization
FOS	Fructooligosaccharides
GAD	Glutamate decarboxylases
GALT	Gut-associated lymphoid tissue
GC	Gas chromato graphy
GOS	Galactooligosaccharides
GRAS	Generally recognized as safe
GT	Gastrointestinal tract
IBD	Inflammatory bowel disease
IMO	Isomaltooligosaccharides
LAB	Lactic acid bacteria
LDCs	Low-digestible carbohydrates
LOS	Lactulose
MBC	Minimum bactericidal concentration
MRS	de Man, Rogosa and Sharpe
MHB	Muller Hinton broth
MIC	Minimum inhibitory concentration
NCFCS	Neutralized cell-free culture supernatant

ABBREVIATIONS AND SYMBOLS (Continued)

NDOs	Non-digestible oligosaccharides
PDX	Polydextrose
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
RS	Resistant starch
SCC	Short-chain carbohydrates
SCFA	Short-chain fatty acids
UC	Ulcerative colitis
XLD	Xylose lysine deoxycholate
XOS	Xylooligosacharides

CHAPTER 1

INTRODUCTION AND LISTERATURE REVIEW

Introduction

Human gastrointestinal tract harbors a hundreds of different types of microorganism including bacteria, fungi and protozoa (Grimoud *et al*., 2010). The quantity of living bacteria which compose the human microbiota can range from 10^{11} to 10^{12} CFU g^{-1} of luminal content and contain up to 500 different species (Candela *et al*., 2005). These microorganisms served as numerous important functions for its host through its metabolic activities and physiological regulation such as promotion of nutrient absorption, synthesis of bioactive compounds, improvement of intestinal barrier function, motility, resistance to pathogens or modulation of the immune system (Palmer *et al*., 2007; Grimoud *et al.*, 2010). Overall, a balanced gut microbiota composition confers benefits to the host, while microbiota imbalances are associated with metabolic and immune-mediated disorders (Laparra and Sanze, 2010). Alteration of the microbiota may cause some direct or indirect digestive pathology like infectious diseases and chronic inflammation, metabolic disorders or atopic diseased (Grimoud *et al*., 2010). One approach to health maintenance, disease control restore the intestinal ecosystem is the use of probiotic and prebiotic (Mandadzhieva *et al*., 2011). Probiotics are defined as live microorganisms which confer a health benefit, promote or support a beneficial balance of the autochronous microbial population of the gastrointestinal tract (Holzapfel and Schillinger, 2002; Picard *et al.*, 2005). Prebiotics are defined as a non-digestible food ingredient that beneficially affects host by selectively stimulating growth and/or activity of one or a limited number of bacteria in the colon and probiotic bacteria (Holzapfel and Schillinger, 2002; Young *et al.*, 2003; Geier *et al*., 2007). Prebiotics may also aid survival of probiotic organisms in gastrointestinal tract (Capela *et al.*, 2006). The combination of probiotic and prebiotic is so called synbiotic. Potentially synbiotic may have benefits greater than that of the probiotic and prebiotic alone, because the prebiotic may enhance the growth, colonization, or activation of the probiotic species (Geier *et al*., 2007; Furrie *et al*., 2005).

In healthy infants breast-feeding induces the development of a microbiota rich in *Bifidobacterium* spp. (Liévin *et al*., 2000). In contrast, colonization of other anaerobes in addition to bifidobacteria and by facultatively anaerobic bacteria is often described in bottle-fed infant (Sakata *et al*., 2005). Lactic acid bacteria isolated from the feces of newborn babies are often able to survive a low pH and a relatively high bile salt concentration with little or no decrease in viable cell population (Park *et al.,* 2002). *Lactobacillus* spp. isolated from the feces of breast-fed infants have also shown resistance to gastric juice and bile salts and the ability to adhere to Caco-2 cells and to elicit antibacterial activity against both Gram-positive and negative potential pathogens (Park *et al.,* 2002).

Naturally occurring prebiotic oligosaccharides are found in many sources such as plants (fructooligosaccharides, inulin, soybean oligosaccharides), animal skeleton (chitin, chitosan and chitosan oligosaccharides), human's and cow's milk (galactooligosaccharides). More than 36,000 plants worldwide containing fructo-oligosaccharides and inulin are most popular substances used as food supplements in various human diets, which are usually stored in organs such as bulbs, tubers and tuberous roots of monocotyledonous and dicotyledonous families such as *Liliaceae*, *Amaryllidaceae*, and *Compositae* (Kaur and Gupta, 2002). The edible parts of plant which common consumed in Thailand have been reported for their prebiotic properties (Moongngarm *et al*., 2011; Wichienchot *et al*., 2011).

In this study, feces of healthy infant were used as a source for isolation probiotic bacteria in this study. Edible plant (okra, mung bean, gros michel, saba) which recognized as common foods consumed in Thailand were evaluated for their prebiotic property. The proper probiotic and prebiotic were combined to develop synbiotic product. Thereafter, the synergistic effect of developed synbiotic product on human gut microflora and *Salmonella enterica* subsp. *enterica* SA2093 in challenging system under simulated proximal region of human gastrointestinal tract were investigated.

Literature review

1. Human gastrointestinal microflora

In the human gastrointestinal tract, there exists variability in bacterial number and populations among the stomach, small intestine and colon (Fooks *et al.*, 2002). The population average 10^{10} -10^{11} bacteria g^{-1} and is dominated by Gram-positive bacteria such as *Lactobacillus, Streptococcus* and bifidobacteria. Three groups of bacteria can be recognized as beneficial, potentially pathogenic and pathogenic bacteria (Westerbeek *et al*., 2006). After the mouth, colonization is markedly influenced, in part by luminal pH, and by progressively slower transit of food materials towards the colon. The movement of digesta through the stomach and small intestine is rapid (ca. 4-6 h), when compared with a typical colonic transit time of around 48-70 h for adults (Vernazza *et al*., 2006). This allows the establishment of a complex and relatively stable bacterial community in the large intestine. The human large intestine consists of the caecum, ascending colon, transverse colon, descending colon, sigmoid colon and rectum (Vernazza *et al*., 2006) (Figure 1). The bacteria concentration in the upper bowel is sparsely populated, and bacterial concentration gradually increase from the reaching 10^{11}-10^{12} colony-forming units (CFU g^{-1}) in the colon (Figure 2). The colon represents the main site of microbial colonization, providing residence for more than 500 different species of bacteria (Leahy *et al.*, 2005). A complex microbiota consisting of facultative and strict anaerobe including *Streptococcus, Bacteroides, Lactobacillus, Bifidobacterium, Eubacterium, Clostridium, Peptococcus, Peptostreptococcus, Ruminococcus, Escherichia, Enterobacter, Enterococcus, Klebsiella, Proteus* and yeasts (Vaughan *et al*., 2000; Isolauri *et al*., 2004; Leahy *et al*., 2005; Westerbeek *et al*., 2006). The colonic microflora is capable of responding to anatomical and physiochemical variations that are present. The right or proximal colon is characterized by a high substrate availability (due to dietary input), a pH of around 5.5-6.0 (from acids produced during microbial fermentation) and a more rapid transit than the distal region. The left or distal area of the colon has a lower concentration of available substrate, the pH is approximately 6.5-7.0 and bacteria grow more slowly. The proximal region tends to be a more saccharolytic environment than the distal gut, the distal gut having higher bacteria is thought to be present in the large intestine (Guarner and Malagelada, 2003).

1.1 Intestinal colonization

The neonatal period is crucial for intestinal colonization (Gronlund *et al.*, 1999). Microbial colonization and development in the gastrointestinal tract of newborn infant begins immediately after birth and occur within a few days (Guarner and Malagelada, 2003; Isolauri *et al.*, 2004; Leahy *et al.*, 2005; Westerbeek *et al.*, 2006). Infants born vaginally apparently acquire their gut flora from maternal vaginal and fecal flora, but the environment also contributes (Gronlund *et al.*, 1999). Natural delivery will expose the child to the maternal vaginal and intestinal microbiota, comprising mainly *Lactobacillus, Bacteriodes, Peptostreptococcus* and *Peptococcus* which constitute the initial source of bacteria colonizing in the intestine of the newborn subsequently (Islauri *et al*., 2004). For the colonization of infants born by cesarean delivery (CD), the environment is extremely important. Likewise, if infants are separated from their mothers for long periods after birth, the environment becomes an important source of colonizing bacteria (Gronlund *et al.*, 1999). Gut colonization is delayed in infants born by CD, and intestinal colonization is consequently abnormal for several weeks.

1.2 Factors influencing the composition of the gut microbiota

A number of different factors are able to affect the composition of the colonic microbiota (Fooks *et al.*, 1999) (Table 1). The diet may exert a major effect on the composition and activity of the gut microbiota. Breast-fed infant tend to contribute higher levels of bifidobacteria than formula fed infant. Bifidobacteria can range from 60 up to 90% of total faecal microbiota in breast fed-infant (Isolauri *et al.*, 2004; Vaughan *et al.*, 2002). The high flow of the contents in the upper part of the gastrointestinal tract does not allow for the accumulation of a large number of microbiota. In the lower part of the gastrointestinal tract, the flow of the digesta become slower and its composition is less antimicrobial substance, supporting the establishment of larger microbiota. Because of the anaerobicity of the lower gastrointestinal tract, anaerobic microbes start to outnumber the aerobic one (Isolauri *et al.*, 2004).

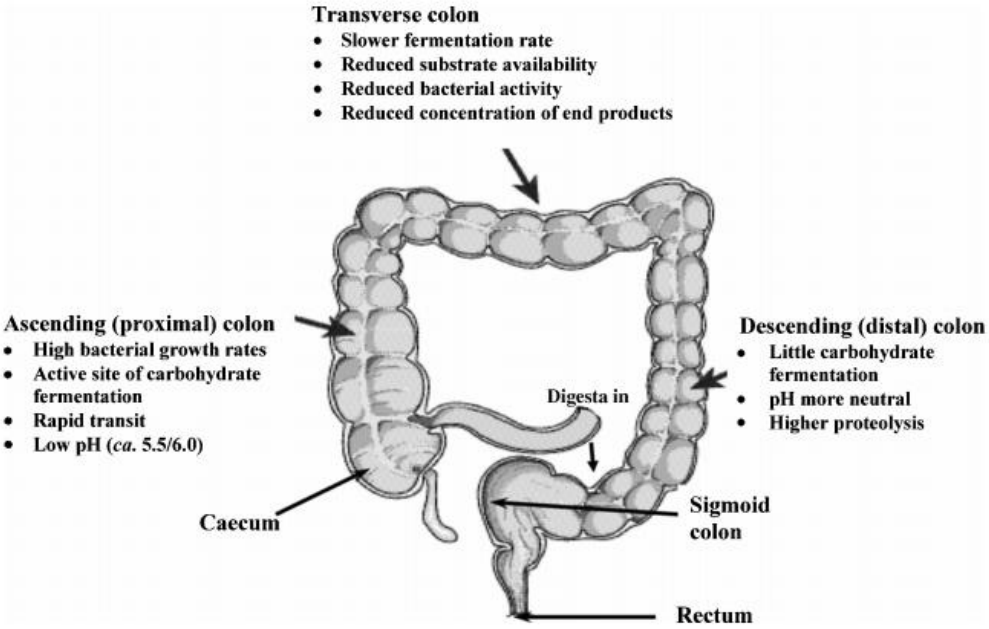


Figure 1. Regions of the human large intestine with corresponding bacterial activities and physiological differences.

Source : Vernazza *et al*. (2006)

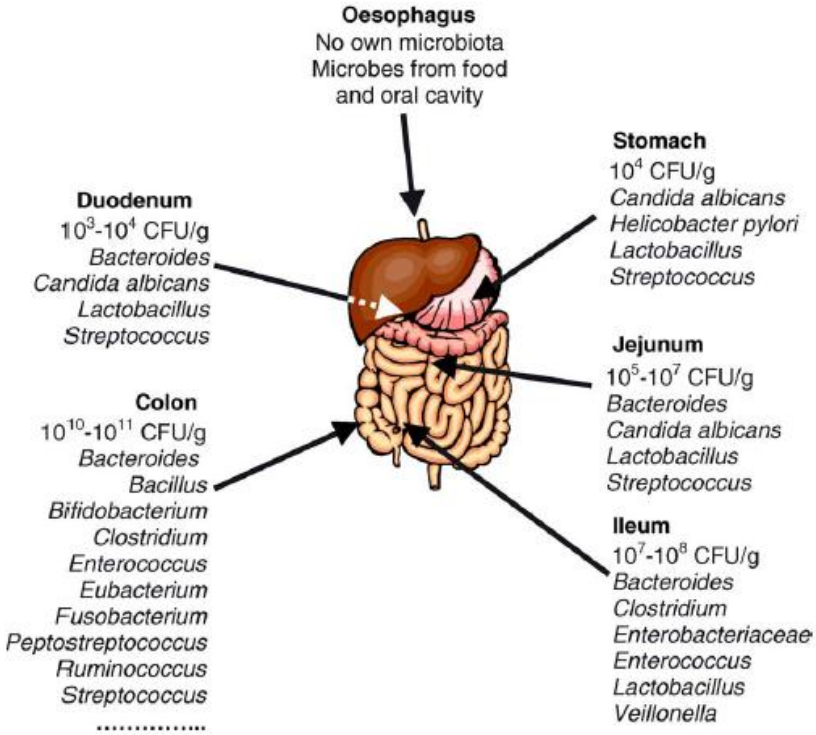


Figure 2. The numerically dominant microbial genera in the human gastrointestinal tract.

Source : Isolauri *et al*. (2004)

Table 1. Examples of factors which may affect the composition of the human gut microflora.

Type of feeding
Amount, chemical composition and availability of growth substrates
Availability of colonization sites
Immunological interactions
Individual fermentation strategies by the bacteria
Intestinal transit time
Gut pH
Redox potential
Availability of inorganic electron acceptors
Production of bacterial metabolites
Presence of antimicrobial compounds
Xenobiotic compounds
Age of the host
Peristalsis

Source : Fook *et al*. (1999)

1.3 Metabolic functions

The metabolic activity of the intestinal microbiota is involved in the fermentation of non-digestible dietary residues and endogenous mucin produced by the epithelia (Guarner and Malagelada, 2003) (Figure 3). Gene diversity in the microbial community provides various enzymes and biochemical pathways that are distinct from the host's own constitutive resources. Overall outcomes of this complex metabolic activity are recovery of metabolic energy and absorbable substrates for the host, and supply of energy and nutritive products for bacterial growth and proliferation. Fermentation of carbohydrates is a major source of energy in the colon. Non-digestible carbohydrates include large polysaccharides (resistant starches, cellulose, hemicellulose, pectins, and gums), some oligosaccharides that escape digestion, and unabsorbed sugars and alcohols. The metabolic endpoint is generation of short-chain fatty acids (Cummings *et al.*, 1987). Fermentation of different types of oligosaccharides is beneficial to the host as it provides additional energy in the form of short-chain fatty acids, butyric acid, acetic acid and propionic acid are the main short-chain fatty acids also involed in the salvage of energy (Leahy *et al.*, 2005). Butyric acid is a main energy source for the intestinal epithelium and it is important in

maintaining mucosal health in the colon (Isolauri *et al*., 2004). Furthermore, fermentation product of several numbers of the intestinal microbiota include ethanol, lactate, succinate, formate, valerate, caproate and vitamin such as vitamin K and vitamin B (Fooks *et al.*, 1999; Leahy *et al.*, 2005) and gases (H_2, CO_2 and CH_4) (Blaut, 2002).

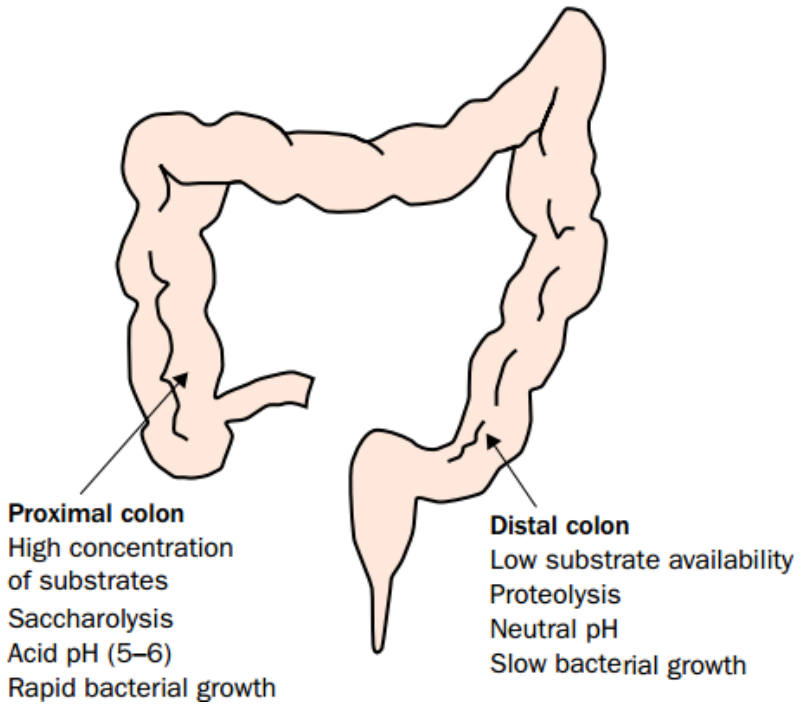


Figure 3. Fermentation in the colon**.**

Source : Guarner and Malagelada (2003)

2. Probiotic

2.1 Probiotic concept

There are many definitions about probiotic (Ouwehand *et al.*, 2002). Probiotics are living microorganisms which upon ingestion in certain number exert health benefit beyond inherent basic nutrition.

Probiotics were originally used to influence both animal and human health through intestinal microbiota alteration (Dunne *et al*., 2001).

Probiotics are live microorganisms, which when consumed in adequate amount; confer a health effect on the host (Sanders, 2008).

Probiotics are microorganisms promoting the growth of other microorganism or microorganism that promote or support a beneficial balance of the autochthonous microbial population of the gastrointestinal tract (GT) (Holzapfel *et al*. 2002).

Probiotics bacteria are frequently used as the active ingredient in functional foods such as bio-yoghurts, dietary adjuncts and health-related products (Prasad *et al.*, 1999). Most probiotic microorganisms belong to *Lactobacillus* sp, *Bifidobacterium* sp, *Enterococcus* sp., *Saccharomyces, Bacillus* sp. and *Clostridium butyricum* especially *Lactobacillus acidophilus* and *Bifidobacterium* sp. are purposed to exert health promoting or probiotic effects in humans and animals.

2.2 Selection criteria for probiotic miroorganism

According to recommendations of FAO/WHO, probiotics must be able to exert their benefits on the host through growth and/or activity in the human body. It is the ability to remain viable at the target site and to be effective, that should be verified for each potentially probiotic strain. There is a need for refinement of *in vitro* tests to predict the ability of probiotics to function in humans (Heczko *et al.*, 2006). It is clear that the selection of new strains provides an exciting challenge both to science and industry. However, even considering that probiotic microorganisms are claimed to promote health, the mechanisms involved have not been fully elucidated yet. Approaches for selection of an ‘‘ideal’’ strain are therefore still difficult and indeed require considerable resources (Holzapfel *et al.*, 1998, Holzapfel and Schillinger, 2002). Selection criteria for probiotic microorganisms include:

2.2.1 Probiotic strain must be safe (Generally regared as safe; GRAS)

The safety and non-pathogenicity of a new strain is considered of major importance. The assessment and proof of a “safe” or “GRAS” strain, without a provious “history of safe use”, has been the topic of controversial discussions in recent years. Approaches for assessing the safety of probiotic and starter strains have been recommended and implied as following (Holzapfel and Schillinger, 2002).

2.2.2.1 Characterization of the genus, species and strain and its origin which will provide an initial indication of the presumed safety in relation to known probiotic and starter strains.

2.2.2.2 Studies on the intrinsic properties of each specific strain and its potential virulence factors.

2.2.2.3 Studies on adherence, invasion potential and the pharmacokinetics of the strain, and

2.2.2.4 Studies on interactions between the strain, intestinal and mucosal microflora, and the host.

2.2.2 Resistance to gastric acidity

More than two liters of gastric juice is secreted each day, with a pH as low as 1.5 providing a barrier to the entrance into the gut of bacteria (Morelli, 2000). The secretion of gastric acid constitutes a primary defense mechanism against most ingested microorganisms. The probiotic bacteria will need to survive the highly acidic gastric juice if they are to reach the small intestine in a viable state (Cotter and Hill, 2003). The acid tolerance of probiotic lactobacilli and bifidobacteria has been widely reported (Duune *et al*., 2001; Vernazza *et al*., 2006; Masco *et al*., 2007).

The tree main systems involved in acid tolerance in lactic acid bacteria, i.e., the H^+-ATPase proton pump, the arginine deiminase system (ADI) and the glutamate decarboxylase system (GAD) (Cotter and Hill, 2003).

2.2.2.1 F_1F_0-ATPase

The multisubunit F1Fo-ATPase links the production of ATP to the transmembrane proton motive force (PMF) and either generate ATP at the expense of PMF established by respiring cells or generate a PMF using ATP produced by fermentative substrate-level phosphorylation. The PMF can facilitate the extrusion of protons from the cell cytoplasm, resulting in a drop of intracellular pH. The membrane-embedded F_0 complex, composed of subunits a, b, and c, has proton-translocation activity, although its channel activity is increased by coexpression of some F_1 proteins, suggesting a possible role for an F_1 protein in the assembly or gating of the channel. The peripherally bound F_1 complex, consisting of subunits α, β, γ, δ, and ε has ATPase activity when it is released from the membranes and catalyzes the coupled interconversion of proton translocation and ATP synthesis or hydrolysis when it is complexed to the Fo complex in sealed membranes. The ATPases from the acid tolerance bacteria are less sensitive to low pH, which is also the explanation for the acid resistance of the *Leuconostoc oenos* mutant LoV8413. In accordance with the above observation, the acid sensitivity of *Lactococcus lactis* subsp. *lactis* C2 mutant and the *Lactobacillus helveticus* mutant CPN4 were explained by reducing ATPase activity at low pH, and thus the variations in acid tolerance in all of the above

examples can be attributed to their relative ATPase activities at low pH (Cotter and Hill, 2003).

2.2.2.2 Arginine deiminase system (ADI)

The arginine deiminase system was found to function in protecting bacterial cells against the damaging effects of acid environments (Casiano-Colón and Marquis, 1988). The ADI catabolizes arginine to ornithine, NH_3, and CO_2. The increased acid tolerance of bacterial cell results from the production of NH_3, which combines with protons in the cytoplasm to produce NH_4^+, raising the internal pH. The ADI system has been identified in a number of lactic acid bacteria (Casiano-Colón and Marquis, 1988; Cotter and Hill, 2003). A little amount of arginine (2.9 mM) added to acidified suspensions of *Streptococcus sanguis* at pH of 4.0 resulted in ammonia production and protection against killing (Casiano-Colón and Marquis, 1988). As well, a low supply of arginine (6 mM) during sourdough fermentation by *L. sanfranciscensis* CB1 enhanced cell growth, cell survival during storage at 7°C, and tolerance to acid environmental stress and favored the production of ornithine, which is an important precursor of crust aroma compounds (Angelis *et al*., 2002).

2.2.2.3 Glutamate decarboxylase system (GAD)

Almost 60 years ago, it was proposed that amino acid decarboxylases function to control the pH of the bacterial environment by consuming hydrogen ions as part of the decarboxylation reaction. Examples of these are lysine, arginine, and glutamate decarboxylases (GAD), which operate by combining an internalized amino acid (lysine, arginine, or glutamate) with a proton and exchanging the resultant product for another amino acid substrate. Thus, an extracellular amino acid is converted to an extracellular product, but the consumption of an intracellular proton results in an increase in intracellular pH. Of the three systems mentioned, only the GAD system has been associated with pH control by Gram-positive cells (Cotter and Hill, 2003).

2.2.3 Bile acid resistance

The ability to survive the action of bile salts is an absolute need of probiotic bacteria, and it is generally included among the criteria used to select potential probiotics strains (Morelli, 2000). Bile acids are synthesized in the liver from cholesterol, stored and concentrated in gall bladder and released into the duodenum in the conjugated form (500-700 ml/d) (Dunne *et al.*, 2001). Bile functions as a biological detergent that emulsifies and

solubilizes lipids, thereby playing an essential role in fat digestion (Begley *et al*., 2006). To increase the solubility, the hydrophobic steroid nucleus is conjugated with either glycine (glycoconjugated) or taurine (tauroconjugated) prior to secretion. The conjugated bile can be transformed to deconjugation form. Both conjugated and deconjugated bile acids exhibit antibacterial activity, primarily through the dissolution of bacterial membranes (Begley *et al*., 2006). Deconjugated bile acid has decreased its solubility and diminished detergent activity and maybe less toxic to bacteria in the intestine than conjugated bile (Moser and Savage, 2001). Deconjugation is catalyzed by bile salt hydrolase (BSH) enzymes (EC 3.5.1.2.4), which hydrolyze the amide bond and liberate the glycin/taurine moiety from the steroid core (Begley *et al*., 2006). BSHs are detergent shock proteins that protect the bacteria that produce them from the toxicity of bile acid in the gastrointestinal tract (Moser and Savage, 2001). BSH from human intestinal lactobacilli generally have higher affinity for glycine conjugates (Ridlon *et al*., 2006) or the higher affinity of BSHs for glycine conjugates may have evolved because glycine conjugates are generally higher in propotion (3:1) than taurine conjugates in human bile (Ridlon *et al*., 2006). The presence of BSH had a selective advantage for the bacterium in bile salt rich environments. The BSH activity benefits the bacterium by enhancing its resistance to conjugated bile salts and increasing its survival in the gastrointestinal tract for colonization (Ramasamy *et al*., 2010).

2.2.4 Adherence to intestinal cell lines

The ability to adhere to intestinal mucosa is considered an important selection criterion for lactic acid bacteria (LAB) intended for probiotic use (Klaenhammer and Kullen, 1999). Adhesion to intestinal mucin is regarded as a prerequisite for colonization by microorganisms (Scholz-Ahrens *et al.*, 2001) especially adhesion of probiotics to the intestinal mucosa is also considered important for modulation of the immune system (Perea Velez *et al.*, 2007), balance of intestinal microflora, intestinal bacterial enzyme activity, and stabilization of intestinal permeability (Walker and Duffy, 1998). HT-29 from human colon cancer cells and Caco-2 are human intestinal cell lines expressing morphologic and physiologic characteristics of normal human enterocytes (Morelli, 2000; Neeser *et al.*, 1989) that have been exploited to elucidate the mechanisms mediating enteropathogen adhesion. Adherence is a situation where bacteria adhere firmly to surface by complete physicochemical interactions between them, including an initial phase of reversible physical contact and a time-dependent phase of irreversible chemical

and cellular adherence. There is energy involved in the formation of an adhesive junction between the bacteria and surfaces. The ability to tolerate gastroenteric environment and the adhesive capacity to HT-29 cell among *Bifidobacterium* strains was different. *Bifidobacterium breve* A04 had higher adhesive capability to HT-29 cells *in vitro* and average adhesive bacteria numbers reached 12.8 ± 0.9 for each HT-29 cell (Liu *et al.*, 2007). Two *Lactobacillus* strains, LAP5 and LF33, from swine and poultry, respectively were able to adhere to human intestinal cell lines, such as Int-407 and Caco-2 cells, and to the intestinal epithelium cells isolated from swine, poultry and mouse (BALB/c) (Tsai *et al.*, 2005). Tuomola and Salminen (1999) studied the adhesion of some probiotic and dairy *Lactobacillus* strains to Caco-2 cell cultures. *Lactobacillus casei* (Fyos) was the most adhesive strain and *L. casei* var. *rhamnosus* was the least adhesive strain.

2.2.5 Antimicrobial activity

LAB strains commonly produce antimicrobial substance(s) with activity against the homologous strain, but LAB strains also often produce microbicidal substances with effect against gastric and intestinal pathogens and other microbes. This could be the mechanism behind reports that some probiotic strains inhibit or decrease translocation of bacteria from the gut to the liver (Ljungh and Wadstrom, 2006). *In vitro* experimental studies have demonstrated that the selected lactic acid strains showed effective against diarrhoeagenic bacteria by producing several metabolic compounds including organic acids, fatty acids, hydrogen peroxide, diacetyl and bacteriocin (Dunne *et al.*, 2001; Servin and Coconnier, 2003).

2.2.5.1 Organic acid

Organic acid levels and types of organic acids produced during the fermentation process depend on LAB species or strains, culture composition and growth conditions (Ammor *et al.*, 2006). Production of lactic acid, the major metabolite of LAB, is responsible for the associated decrease in pH, which may be sufficient to antagonize many microorganisms. It has been proposed that the low external pH causes acidification of the cell cytoplasm, while the undissociated acid, acts by collapsing the electrochemical proton gradient, or by altering the cell membrane permeability which of substrate transport systems of susceptible bacteria, leading to bacteriostasis and eventual death (Ammor *et al.*, 2006, Soomro *et al.*, 2002; Tome *et al.*, 2006). Lactic acid is the major organic acid of LAB fermentation where it is in equilibrium with its undissociated and dissociated forms, and the

extent of the dissociation depends on pH (Ammor *et al.*, 2006). Spent culture supernatant (SCS) of the probiotic *Lactobacillus rhamnosus* GG under the growth conditions produced lactic acid to exert antibacterial activity against *Salmonella typhimurium* (De Keersmaecker *et al.*, 2006).

2.2.5.2 H_2O_2

Production of H_2O_2 is considered to be beneficial for food preservation and prevention of pathogen implantation and growth. H_2O_2 is generated by some micro-organisms growing aerobically and usually generated by lactobacilli present in a healthy vagina, but is mostly absent in women with bacterial vaginosis (Batdorj *et al.*, 2007; Kaewsrichan *et al.*, 2006). Many reports have demonstrated the production of H_2O_2 by LAB. Some lactobacilli have been reported to inhibit the growth of food spoilage bacteria and pathogens in associative cultures and others have been reported to present an activity towards various human intestinal and vaginal pathogens (Batdorj *et al.*, 2007). Hydrogen peroxide is produced by LAB in the presence of oxygen as a result of the action of flavoprotein oxidases or nicotinamide adenine dinucleotide (NADH) peroxidase (Ammor *et al.*, 2006). The antimicrobial effect of H_2O_2 may result from the oxidation of sulfhydryl groups causing denaturing of a number of enzymes, and from the peroxidation of membrane lipids thus increasing membrane permeability (Ammor *et al.*, 2006). Production of H_2O_2 was studied under aerated and nonaerated culture conditions. The amount of H_2O_2 in the culture supernatant increased during bacterial growth and reached a maximum (5.12 mmol l^{-1}) at the early stationary phase under aerated conditions (agitated cultures) but was not detected in the culture performed without agitation (Batdorj *et al.*, 2007).

2.2.5.3 CO_2

Carbon dioxide is mainly produced by heterofermentative LAB (Ammor *et al.*, 2006). Hydrogen peroxide producing activity has been suggested as the main protective factor from vaginal pathogens (Nguyen *et al.*, 2007). However, CO_2 may play a role in creating an anaerobic environment which inhibits enzymatic decarboxylations, and the accumulation of CO_2 in the membrane lipid bilayer may cause a dysfunction in permeability. CO_2 can effectively inhibit the growth of many food spoilage microorganisms, especially Gram-negative psychrotrophic bacteria. The degree of inhibition by CO_2 varies considerably between the organisms. CO_2 at 10% (v/v) could lower the total bacterial counts by 50% (v/v), and at 20–50% it had a strong antifungal activity (Ammor *et al.*, 2006).

2.2.5.4 Bacteriocin

Bacteriocins (as colicins) were originally defined as bacteriocidal proteins characterized by lethal biosynthesis (Chen and Hoover, 2003). They are defined as ribosomally synthesized, secreted, bactericidal peptides, and are produced by some micro-organisms in all major lineages of Eubacteria and Archaebacteria (Millette *et al.*, 2007). Bacteriocin production could be considered as advantageous to the producer as, in sufficient amounts; these peptides can kill or inhibit bacteria comting for the same ecological niche or the same nutrient pool (Deegan *et al.*, 2006). It can inhibit the growth of Gram-positive pathogenic and spoilage bacteria as well as yeasts besides, it has been reported that bacteriocins also inhibit the growth of some Gram-negative species (Topisirovic *et al.*, 2006). Most bacteriocin-producing LAB have been isolated from fermented food stuffs but bacteriocin-producing human strains of *Lactobacillus* or *Bifidobacterium* have been isolated from the human intestine, stool or vaginal tract (Millette *et al.*, 2007; Toure *et al.*, 2003). *Lactobacillus salivarius* UCC118, a recently sequenced and genetically tractable probiotic strain of human origin, produces a bacteriocin in vivo that can significantly protect mice against infection with the invasive foodborne pathogen *Listeria monocytogenes* (Corr *et al.*, 2007).

2.2.6 Antibiotic resistance

Many strains of lactobacilli are naturally resistant to vancomycin. It is accepted that antibiotic nonsusceptibility/resistance is not, in itself, a hazard unless it renders the probiotic untreatable in rare cases of infection or unless it can be transferred to potential pathogens for which resistance could have therapeutic consequences. The vancomycin resistance genes of *Lactobacillus* species appear to be chromosomally located and are not easily transferable to other genera. Vancomycin would not be used for the treatment of a case of lactobacillemia. When used as probiotics, selected strains should be susceptible to major antibiotics. Currently, it is difficult to interpret studies of gene transfer *in vivo*, and the methods involved need to be further developed. The focus should be on transfer to *Enterococcus* species and *Staphylococcus aureus*, for which there are potential clinical consequences, rather than on homologous gene transfer (Borriello *et al.*, 2003). The resistance of heterofermentative and facultative heterofermentative lactobacilli to vancomycin is thought to be intrinsic, due to the presence of *D*-Ala-*D*-lactate in their peptidoglycan instead of the normal dipeptide *D*-Ala-*D*-Ala. Cell wall impermeability is

probably the main mechanism of resistance to the cephalosporin cefoxitin, since lactobacilli, like many other lactic acid bacteria, lack cytochrome-mediated electron transport (Delgado *et al.*, 2007). Intrinsic resistance to certain antimicrobial agents (cefoxitin, metronidazole and vancomycin) was observed in most lactic acid bacteria from the feces of healthy human isolates, but atypical resistances to erythromycin, clindamycin, or tetracycline were also found in 5 strains. Undesirable traits such as *alpha*-chymotrypsin or *N*-acetyl-*beta*-glucosaminidase activities were not detected, but low beta-glucuronidase and moderate beta-glucosidase activities were recorded in 2 strains (Delgado *et al.*, 2007). *Lactococcus lactis* ssp. *lactis* HV219 sensitive to most antibiotics tested, but resistant to amikacin, ceftazidime, nalidixic acid, metronidazole, neomycin, oxacillin, streptomycin, sulphafurazole, sulphamethoxazole, sulphonamides, tetracycline and tobramycin. Ibuprofen, ciprofloxacin, diklofenak and nonoxylol-9 inhibited the growth of strain HV219 (Todorov *et al.*, 2007).

2.3 probiotic effect

There are a variety of proposed beneficial health effects of probiotics; only a few have significant research to back up the claims and will be discussed in this paper. Clinical symptoms that have been reportedly treated or have the potential to be treated with probiotics include diarrhoea, gastroenteritis, irritable bowel syndrome, and inflammatory bowel disease (IBD; Crohn’s disease and ulcerative colitis), cancer, depressed immune function, inadequate lactase digestion, infant allergies, failure-to-thrive, hyperlipidaemia, hepatic diseases (Figure 4).

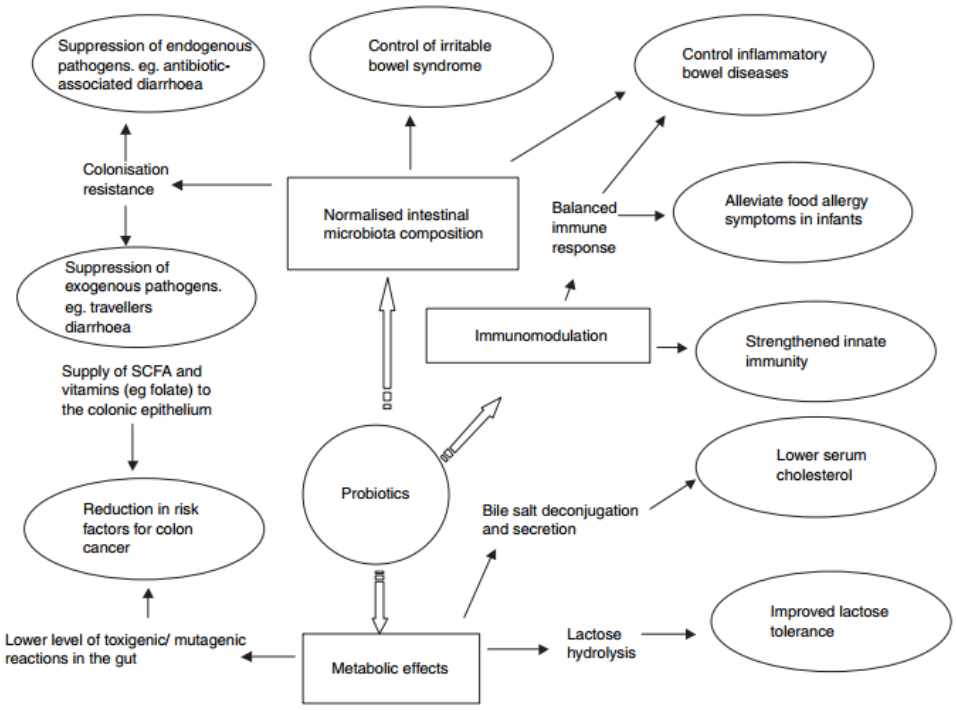


Figure 4. Various health benefits from probiotics consumption.

Source : Parvez *et al.* (2006)

2.3.1 Alleviation of lactose intolerance

Lactose intolerance is a problem for ≤70% of the world's population who have a low amount of intestinal *β*-galactosidase activity and for whom lactose behaves like an osmotic, nondigestible carbohydrate (Leahy *et al.*, 2005). Consumption of lactose leads to an increased osmotic load in the small intestine with subsequent secretion of fluids into the small intestine which leads to loose stools, abdominal bloating, pain, flatulence and occasionally nausea and vomiting when symptoms are severe incurred by lactose are also leading features of irritable bowel syndrome (de Vrese *et al.*, 2001; Leahy *et al.*, 2005; Ouwehand *et al.*, 2002; Szilagyi *et al.*, 2002). It is generally accepted that fermented milk products yogurt containing probiotics bacteria such as *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* have been shown to improve lactose digestion by reducing the intolerance symptoms as well as by slowing orocecal transit (de Vrese *et al.*, 2001; Leahy *et al.*, 2005). This can be explained by the presence of *β*-galactosidase in the bacteria fermenting the milk (de Vrese *et al.*, 2001). Upon ingestion, the bacteria are lysed by bile in the small intestine; the enzyme is released to degrade lactose.

2.3.2 Intestinal infection

Adhesion to epithelial cells is an important step for both pathogenic bacteria and probiotics indicating a potential interaction between them. Adhesion of probiotics to the mucosa has been related to many of the health benefits attributed to probiotics such as antagonism against pathogens by interference mechanisms (Collado and Sanz, 2007e). Ability to inhibit the adhesion of pathogens appears to depend on both the specific probiotic strains and the pathogen tested, indicating a very high specificity. Some of probiotic strains showed high inhibition ability with values of inhibition over 40% for some of the pathogens tested (Collado and Sanz, 2007d). In addition, bifidobacteria have also been examined for their antagonistic activities against other microbial pathogens (Servin and Coconnier, 2003). Several mechanisms of protection have been suggested which are not exclusive. These include the production of various acids, hydrogen peroxide or bacteriocins, the competition for nutrients or adhesion receptors, anti-toxin action and stimulation of the immune system (Leahy *et al.*, 2005). The intakes of high levels of viable bifidobacteria have shown a substantial decrease in *Clostridium* counts as well as an increase in *Bifidobacterium* counts. *Bifidobacteria* have also been shown to exhibit inhibitory effects on many other pathogenic organisms: *B. longum* was reported to provide benefits against the pathogenic challenge of *Salmonella enterica* serova Typhimurium in animal models.

2.3.3 Suppression of cancer

Colorectal cancer (CRC) is one of the most frequent causes of death due to cancer in populations of developed countries who consume ‘Western style diets’ (Pool-Zobel, 2005). Epidemiological studies show that colon cancer is of especially high incidence in the developed western world (Commane *et al.*, 2005). This may be, in part, related to a genetic susceptibility, the high fat low fibre diet typical of western culture is implicated in the aetiology of the disease (Commane *et al.*, 2005). Consumption of large quantities of dairy products such as yoghurt and fermented milk containing *Lactobacillus* or *Bifidobacterium* spp. may be related to a lower incidence of colon cancer (Rafter, 2002). The precise mechanisms by which lactic acid bacteria may inhibit colon cancer are currently unknown. However, mechanisms might include: an alteration of the metabolic activities of intestinal microflora; an alteration of physicochemical conditions in the colon; the binding and degradation of potential carcinogens; quantitative and/or qualitative alterations in the intestinal microflora incriminated in producing putative carcinogen(s) and promoters (e.g.

bile acid-metabolizing bacteria); the production of anti-tumorigenic or antimutagenic compounds; an enhancement of the host's immune response; and effects on host physiology. These potential mechanisms are addressed individually below (Rafter, 2002; 2003).

2.3.4 Digestive aid

It is thought that probiotics help the digestion of food materials. This would be directly related to their viability and ability to colonize effectively (Fooks *et al.*, 1999).

2.3.5 Immune stimulation

The immune system guards the body against foreign substances and protects from invasion by pathogenic organisms (Watzl *et al.*, 2005). The largest immune organ is situated in the gut where continuous exposure to diverse antigens takes place (Watzl *et al.*, 2005). Gut-associated lymphoid tissue (GALT) is first contact that ingested bacteria, including probiotics, have with the immune system (Tuomola and Salminen, 1998). The GALT is part of the mucosal immune systems (i.e. gastrointestinal tract, respiratory tract, oral cavity, urogenital tract and mammary glands) contains about 60% of all lymphocytes in the body (Tuomola and Salminen, 1998; Watzl *et al.*, 2005). The absence of the intestinal microflora antigen transport is increased indicates that the gut microflora is an important constituent in the intestines defence barrier (Ouwehand *et al.*, 2002). In affecting the development of gut-associated lymphoid tissue at an early age the gut microflora directs the regulation of systemic and local immune responsiveness, including hyporesponsiveness to antigens derived from micro-organisms and food (Ouwehand *et al.*, 2002). As treatment, probiotics have so far been used most for gastrointestinal disorders, especially antibiotic-associated diarrhea but the possibility that they can alter extraintestinal immune function is suggested by the efficacy of feeding *Lactobacillus rhamnosus* in improving eczema in children effect that might be related to its induction of increased IFN-γ production (Yoo *et al.*, 2007) or the ability of the organisms to alter intestinal permeability, as well as a possible direct effect on the gut-associated lymphoid tissue, the largest lymphoid organ in the body (Young and Huffman, 2003).

2.3.6 Food allergy

Food allergy is defined as an immunologically mediated adverse reaction against dietary antigens. Food allergy can affect several organ systems, the symptoms commonly arising from the gut, skin and respiratory tract. Despite the wide spectrum of clinical manifestations, there are at least two prerequisites for the development of food

allergy: dietary antigens must penetrate the intestine's mucosal barrier, and the absorbed antigens must cause harmful immune responses. In food allergy, intestinal inflammation and disturbances in intestinal permeability and antigen transfer occur when an allergen comes into contact with the intestinal mucosa (Tuomola and Salminen, 1998). Probiotic bacteriotherapy has great potential in controlling the allergic inflammation associated with food allergy (Kirjavainen *et al.*, 1999). The possible mechanisms by which probiotics alleviate the inflammatory response in food allergy include their potentially effect on the nonimmunologic and immunologic defense barrier of the gut and modified degradation of food allergens (Kirjavainen *et al.*, 1999). By being partly attributable to enhanced production of anti-inflammatory cytokines, e.g. interleukin-10 and transforming growth factor-*β*, and partly due to control of allergic inflammation in the gut (Isolauri *et al.*, 2001, Ouwehand *et al.*, 2002) or producing antimicrobial substances and coaggregation with pathogens, probiotics can normalize the intestinal flora and thereby alleviate inflammation, normalize permeability, and reduce the permeation of food antigens in hypersensitive subjects (Kirjavainen *et al.*, 1999). Serum total IgE levels were found to be significantly lower in those subjects habitually eating yogurt and/or fermented milk drinking, in comparison with those who do not habitually eat such fermented milk foods, these findings supported the idea that intestinal bacteria such as lactic acid bacteria and bifidobacteria involve in regulation of allergy development (Enomoto *et al.*, 2006) and can have an inhibitory impact on allergic inflammation (von der Weid *et al.*, 2002).

3. Bifidobacteria

Bifidobacteria are a genus of anaerobic bacteria and a form of probiotic that is thought to have health-promoting properties from human. They are Gram-positive, heterofermentative, non-motile, non-spore forming rods catalase-negative, non-gas producing with bifidus morphology which were originally termed *Bacillus bifidus* (Gagnon *et al.*, 2004; Lievin *et al.*, 2000). It has various shapes, including short rods, curved rods, club-shaped rods, and bifurcated Y-shape rods (Leahy *et al.*, 2005; Lievin *et al.*, 2000). The morphotype of bifidobacteria cells may vary depending on species; culture conditions (Klijn *et al.*, 2005). Nonetheless, there was still not a taxonomic consensus for this new genus and for much of the 20th century. They were classified as member of the genus *Lactobacillus*, because of their rod-like shape and obligate fermentative characteristic. However the

accumulation of studies detailing DNA hybridization, G+C content and unique metabolic capabilities resulted in the resurrection of the *Bifidobacterium* genus (Leahy *et al.*, 2005). Normally inhabit of the human and animal colon newborns, especially those that are breast-fed. They are more numerous in the infant gut, where they form up to 91% of the total microflora in breast-fed babies and up to 75% in formula-fed infant (Hadadji *et al.*, 2005). Goncharova *et al*. (1989) reported that bifidoflora constitute 85-95% of the large intestine of children under 1 year old. Bifidobacteria are considered to be beneficial bacteria, and they are used in the preparation of probiotic products. These bacteria are believed to provide several healths, nutritional and therapeutic benefits to human hosts including reduction of blood cholesterol, improvement of lactose utilization in malabsorbers deconjugation of bile acids and increase immunity in animal hosts. They are considered essential for maintaining healthy equilibrium between beneficial and potentially harmful microorganisms in the gastrointestinal tract. Gueimonde *et al*. (2007) reported that bifidobacteria play important role in the pathogenesis of colorectal cancer, diverticulitis and inflammatory bowel disease. As well, *Bifidobacterium animalis* can protect the intestine from alterations induced by zinc deficiency, suggesting that this bacterium may play a role in intestinal mucosal defense (Mengheri *et al.*, 1999).

4. Lactobacilli

Lactobacilli are Gram-positive, generally anaerobic, non-spore-forming rods. Some species present as coccobacilli, bent rods, coryneform or thread-like. Lactobacilli are also generally associated with the infant gut microbiota, although not predominant organisms in the flora are isolated throughout the gastrointestinal tract of healthy humans (Olano-Martin *et al.*, 2000). There is, however, host specificity in colonization by individual species; for example, *Lactobacillus acidophilus, L. fermentum,* and *L. plantarum* are commonly found in the feces of humans (Conway *et al.*, 1987). They can be easily isolated from infant feces in those culture media suited for the growth of lactic acid bacteria and are generally considered as an important part of the intestinal microbiota. The importance properties for lactobacilli to be effective probiotic are the ability to adhere to cells, exclude or reduce pathogenic adherence, persist and multiply, produce acids, hydrogen peroxide and bacteriocins antagonistic to pathogenic growth, be safe and therefore noninvasive, noncarcinogenic and nonpathogenic, resist vaginal microbicides and coaggregate and form a

normal balanced flora (Reid, 1999). Oral administration of *Lactobacillus salivarius* Ls-33 had a significant preventive effect on colitis in mice (Daniel *et al.*, 2006). *Lactobacillus* administration has been studied as a means of treating and preventing disease. *Lactobacillus acidophilus* appears to be involved in beneficial antagonistic and cooperative reactions that interfere with establishment of pathogens in the gastrointestinal tract. The mechanisms of action are believed to involve competitive exclusion and production of inhibitory substances, including bacteriocins (Reid *et al.*, 1990).

5. Prebiotic

Prebiotics are compounds, usually carbohydrates, which are resistant to direct metabolism by the host and reach the colon and selectively stimulating the growth of beneficial bacteria (Aidera and de Halleux, 2007; Gibson, 1999; Gibson and Roberfroid, 1995) such as bifidobacteria, lactobacilli and eubacteria, considered beneficial for the human host (Aryana and McGrew, 2007; Mussatto and Mancilha, 2007), but not promote potential pathogens such as toxin-producing clostridia, proteolytic bacteriodes and toxigenic *Escherichia coli* (Manning and Gibson, 2004). Growth of feacal bifidobacteria was stimulated in infant receiving prebiotic oligosaccharides mixture (90% galacto-oligosaccharides and 10% fructo-oligosaccharides in a concentration of 1 g/d). Such stimulating might protect the newborn from enteric infections (Knol *et al.*, 2005). These resistant short-chain carbohydrates (SCC) referred to nondigestible oligosaccharides or low-digestible carbohydrates (LDCs) provide interesting possibilities for inclusion into conventional food products for their ‘‘bifidogenic’’ effects. Presently, the use of foods that promote a state of well-being, better health and reduction of the risk of diseases have become popular as the consumer is becoming more and more health conscious. In this sense, there has been a lot of attention paid to specific types of diet (Alandera *et al.*, 2001) carbohydrates, namely the non-digestible oligosaccharides (NDOs) (Mussatto and Mancilha, 2007)**.**

5.1 Criteria which allow the classification of a food ingredient as a prebiotics

The criteria used for classification of a food component as a prebiotic are as follows: resistance to digestion, hydrolysis, absorption in the upper part of the gastro-intestinal tract (Fooks *et al.*, 1999; Gibson, 1999; Roberfroid, 2001), fermentation by one or

limited number of potentially beneficial colonic microflora (Gibson, 1999), and most importantly, selective stimulation of growth of one or a limited number of bacteria in the feces (*in vivo* in humans) (Roberfroid, 2001). Resistance to digestion should ultimately be shown in *vivo*. The most adequate model probably is ileostomy patients. To show and quantify hydrolysis and fermentation by colonic microflora, human fecal slurries are a valuable surrogate for colonic content, even though both quantitative and qualitative differences may exist in the microbiota colonizing the different segments of the large bowel (Roberfroid, 2001) and alteration in the composition of the colonic microbiota towards a healthier composition (Fooks *et al.*, 1999).

5.2 Oligosaccharide properties

Any foodstuff that reaches the colon, e.g. non-digestible carbohydrates, some peptides and proteins, as well as certain lipids, is a candidate prebiotic, especially non-digestible carbohydrates seem authentic prebiotics (Fooks *et al.*, 1999). The carbohydrates can be classified according to their molecular size or degree of polymerization (number of monosaccharide units combined), into monosaccharides, oligosaccharides or polysaccharides. According to IUBIUPAC nomenclature, oligosaccharides are defined as sugars containing of 3-10 saccharide units (Mussatto and Mancilha, 2007) or between approximately 2-20 saccharide units (Manning and Gibson, 2004). In addition, based on the physiological properties, the carbohydrates can be classified as digestible or non-digestible. The concept of non-digestible oligosaccharides (NDOs) originates from the observation that the anomeric C atom (C1 or C2) of the monosaccharide units of some dietary oligosaccharides has a configuration that makes their osidic bounds non-digestible to the hydrolytic activity of the human digestive enzymes. The main categories of NDOs avaiable as food ingredients include carbohydrates in which the monosaccharide unit is fructose, galactose, glucose and/or xylose (Figure 5) (Mussatto and Mancilha, 2007).

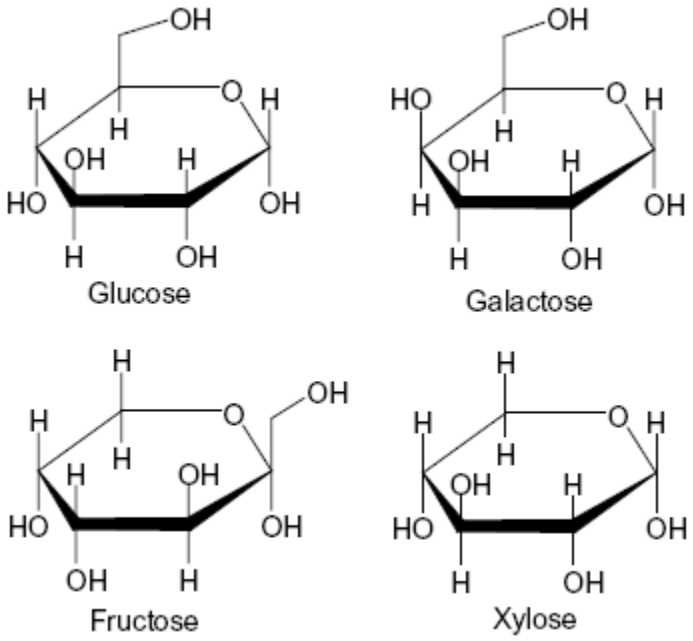


Figure 5. Monosaccharides components of non-digestible oligosaccharides.
Source : Mussatto and Mancilha (2007)

The NDOs are known to promote growth of beneficial bacteria in the colon, mainly the *Bifidobacterium*, and are thus recognized as prebiotics. The majority of candidate prebiotics are oligosaccharides but also include polysaccharides (Table 2) (Blaut, 2002). A number of novel dietary carbohydrates, especially NDOs, have been introduced as functional food ingredients with additional nutritional value during the last few decades. Industrial production processes have been established to extract NDOs from natural sources, by hydrolyzing polysaccharides, and by enzymatic and chemical synthesis from disaccharides substrates. These NDOs are used in a variety of foods not only prebiotic effect but also for their non-cariogenic effect. They represent less sweet sweeteners and enhance physical properties of foods (Sako *et al.*, 1999).

Table 2. Prebiotics and candidate prebiotics.

Compound	Composition	DP*
Xylo-oligosaccharides	β (1→4) linked xylose moieties	2–4
Soybean oligosaccharides	Raffinose (F-Gal-G) and stachyose (F-Gal-Gal) mixtures	3–4
Transgalactosylated oligosaccharides	6' Galactosyllactose	2–8
Palatinose condensates	Enzymatically rearranged sucrose molecules	2–7
Isomaltooligosaccharides	Transgalactosylation of maltose	2–8
Inulin	β (2→1) Fructans	2–65
Oligofructose	β (2→1) Fructans	2–8
Lactulose (Bifiteral®)	Galactosyl-β (4→1) fructose	3–5

DP* Degree of polymerization

Source : Blaut (2002)

5.3 Physicochemical properties

Oligosaccharides are water soluble and mildly sweet, typically 0.3-0.6 times as sweet as sucrose. The sweetness of oligosaccharide product is dependent on the chemical structure and molecular mass of the oligosaccharides present, and the levels of mono- and disaccharides in the mixture (Crittenden and Playne, 1996; Voragen, 1998). Their relatively low sweetness is useful in food production when a bulking agent with reduced sweetness is desirable to enhance other food flavours. Compared with mono- and disaccharides, the higher molecular weight of oligosaccharides provides increased viscosity, leading to improved body and mouth feel. They can also be used to alter the freezing temperature of frozen foods, and to control the amount of browning due to maillard reactions in heat-processed foods. Oligosaccharides provide a high moisture-retaining capacity, preventing excessive drying, and a low water activity, which is convenient in controlling microbial contamination. Many oligosaccharides have also shown to be strong inhibitors of starch retrogradation. The sweetness decreases with longer chain length of oligosaccharides. These low sweetness intensity is quite useful in the various kinds of foods where these of

sucrose is restricted by its high sweetness property (Crittenden and Playne, 1996; Mussatto and Mancilha, 2007).

5.4 Physiological properties

Unlike starch and simple sugars, the currently available food-grade oligosaccharides are not utilized by oral microflora. Consequently, the production of acids or polyglucans (cariogenic compounds) does not occur. Therefore, the NDOs can be used as low cariogenic sugar substitutes in products like confectionery, chewing gums, yoghurts and drinks (Crittenden and Playne, 1996; Mussatto and Mancilha, 2007). Many NDOs are not digested by humans because the human body lacks the enzymes required to hydrolyze the β-links formed among the units of some monosaccharides. Such compounds include carbohydrates where fructose, galactose, glucose and/or xylose are the monosaccharides unit presents. This property makes the NDOs suitable for use in sweet, low-caloric diet foods, and for consumption by individuals with diabetes (Crittenden and Playne, 1996; Mussatto and Mancilha, 2007). Most oligosaccharides are quantitatively hydrolyzed in the upper part of the gastrointestinal tract. The resulting monosaccharides are transported via the portal blood to the liver and, subsequently, to the systemic circulation. Such carbohydrates are essential for health as they serve both as substrates and regulators of major metabolic pathways. Nevertheless, some oligosaccharides present specific physicochemical properties and resist to the digestive process, reaching the caeco-colon as they have been eaten. In the caeco-colon, most (but not necessarily all) of the non-digestible oligosaccharides are hydrolyzed to small oligomers and monomers, which are further metabolized by one, a few, or most of the anaerobic bacteria. Such a metabolic process, known as fermentation, not only serves the bacteria by providing energy for proliferation, but it also produces gases (H_2, CO_2, CH_4), which are metabolically useless to the host, and small organic acids (short-chain fatty acids – SCFA) such as acetate, propionate, butyrate and L-lactate. The amounts and types of SCFA produced in the colon depend on the type of NDO substrate as well as on the composition of the intestinal flora (Sako *et al.*, 1999). The NDOs fermentation in the caecon-colon by the existing colonic bacteria may cause the following effects on the health.

5.4.1 Significant modification of the colonic microflora

Oligosaccharides serve as substrate for growth and proliferation of anaerobic bacteria, mainly bifidobacteria, which inhibit the growth of putrefactive and pathogenic

bacteria present in the caeco-colon. For example, the establishment of a bifidus microflora in the intestines of breast-fed infants has been attributed to the presence of galactose-containing oligosaccharides in human milk. Significant increases of colonic bifidobacterial populations were noted when inulin and oligofructose were added to a controlled diet, and it has been proposed that these changes promote both colonic and systemic health through modification of the intestinal microflora (Jenkins *et al.*, 1999).

5.4.2 Decrease of pH in the colon and consequently

Lower pH values inhibit the growth of certain pathogenic bacterial species while stimulating the growth of the bifidobacteria and other lactic acid species.

5.4.3 Nutrient production

Fermentation of NDOs by the existing colonic bacteria results to production of nutrient such as vitamins of the B complex (B1, B2, B6 and B12), nicotinic and folic acids.

5.4.4 Increase in fecal dry weight excretion

Increasing in fecal dry weight is related to the increased number of bacteria resulting from the extensive fermentation of NDOs.

5.4.5 Constipation relief

The indigestible quality of NDOs means that they have effects similar to dietary fiber, and thus prevent constipation. The end products of NDOs fermentation by colonic bacteria, the SCFA, are efficiently absorbed and utilized by the human colonic epithelial cells, stimulating their growth as well as the salt and water absorption, increasing thus the humidity of the fecal bolus through osmotic pressure, and consequently improving the intestinal motility. Lactulose, which is a substrate for lactic acid bacteria (prebiotic), is effective to treat patients with chronic constipation (Fernandez-Banares, 2006).

5.4.6 Inhibition of diarrhea

Inhibition of diarrhea may be directly related to the possible inhibitory effect of bifidobacteria both on Gram-positive and Gram-negative bacteria.

5.4.7 Increase in absorption of different minerals

Increase in absorption of different minerals, such as iron, calcium, and magnesium, due to the binding sequestering capacity of the NDOs. The minerals that are bound/sequestered and, consequently, are not absorbed in the small intestine reach the colon, where they are released from the carbohydrate matrix and absorbed. The increase on calcium absorption, in particular, reduces the risk of osteoporosis since this mineral

promotes an increase in the bone density and bone mass. The hypotheses most frequently proposed to explain this enhancing effect of NDOs on mineral absorption are the osmotic effect, acidification of the colonic content due to fermentation and production of SCFA, formation of calcium and magnesium salts of these acids, hypertrophy of the colon wall.

5.4.8 Beneficial effect on the carbohydrates and lipids metabolism

Benefit effect on the carbohydrates and lipids metabolism, leading to a decrease in the cholesterol, triglycerides and phospholipids concentration in the blood, reducing thus the risk of diabetes and obesity. Changes in the concentration of serum cholesterol have been related with changes in the intestinal microflora. Some strains of *Lactobacillus acidophilus* assimilate the cholesterol present in the medium, while others appear to inhibit the absorption of cholesterol through the intestinal wall. On the other hand, the changes in lipid metabolism were suggested to be a consequence of a metabolic adaptation of the liver that might be induced by SCFA.

5.4.9 Reduction of cancer risk, mainly the gut cancer

The anticarcinogenic effect appears to be related to an increase in cellular immunity, the components of the cell wall and the extra-cellular components of bifidobacteria. Fecal physiological parameters such as pH, ammonia, *p*-cresol, and indole are considered to be risk factors not only for colon cancer development but also for systemic disorders. It has been demonstrated in a human study that the intake of transgalactosylated disaccharides reduces the fecal pH as well as ammonia, *p*-cresol and indole concentrations with an increase in bifidobacteria and lactobacilli and a decrease in *Bacteroidaceae* populations. These alterations may be considered to be beneficial in reducing the risk of cancer development. A low colonic pH may also aid in the excretion of carcinogens. Prebiotics such as fructans, and probiotics such as Lactobacilli or Bifidobacteria, or a combination of prebiotics and probiotics (synbiotics) are thought to be protective against colon cancer (Femia *et al.*, 2002).

5.5 Natural sources of non-digestible oligosaccharides

NDOs of various types can be found as natural components in milk, honey, fruits and vegetables such as onion, Jerusalem artichoke, chicory, leek, garlic, artichoke, banana, rye, barley, yacon and salsify (Mussatto and Mancilha, 2007). Non-digestible carbohydrates of plant origin include resistant starch as well as non-starch polysaccharides such as cellulose, hemicellulose, pectin and inulin which refer to as dietary fiber (Blaut,

2002). Other examples of naturally occurring non-digestible oligosaccharides are the galactosylsucroses, raffinose and stachyose in soybean and other pulses and leguminous seeds, xylooligosaccharides in bamboo shoots and galactose-containing oligosaccharides in milk, particularly colostrums either in free form or as glycol-conjugates (Mussatto and Mancilha, 2007). Inulin is derived carbohydrate with the benefits of soluble dietary fiber that obtained from several plants such as Jerusalem artichoke, artichokes, chicory, dahlias and dandelions (Lopez-Molina *et al.*, 2005). Gum arabic can reach the large intestine without digestion in the small intestine; it can be categorized as a non-digestible carbohydrate or dietary fiber. It is fermented by intestinal bacteria to SCFA, particularly propionic acid, in the large intestine (Phillipsa *et al.*, 2008). Seeds of legumes, lentils, peas, beans, chickpeas, mallow, composite, and mustard are rich in raffinose oligosaccharides and products derived thereof such as treacles or food-grade molasses (Mussatto and Mancilha, 2007). In addition, there are other sources of natural NDOs. Isomaltulose naturally occurs in honey, sugarcane juice, and products derived such as treacles or food-grade molasses. Cyclodextrins are naturally occurring water-soluble glucans. Moreover, human milk contains more than 130 different oligosaccharides at a concentration of 15–23 g l^{-1} in colostrum and 8–12 g l^{-1} in transitional and mature milk. The carbohydrate chains of almost all oligosaccharides in human milk so far isolated contain lactose at the reducing terminal. Recently, human milk oligosaccharides were shown to be resistant to enzymatic digestion in the upper gastrointestinal tract (Agostoni *et al.*, 2004).

5.6 Prebiotic effect

The prebiotic potential of oligosaccharides such as fructooligo-saccharide, xylooligosaccharide or galactooligosaccharide is based on their selective fermentation by *Bifidobacterium* spp., and to a lesser extent, *Lactobacillus* spp (Kolida *et al.*, 2000).

Lactulose is also manufactured by an isomerization reaction of lactose whose milk and lacto serum are very rich (Kolida *et al.*, 2000, Mussatto and Mancilha, 2007). The average lactose content in milk or milk whey is approximately 4.5%. Theoretically, lactulose can be obtained starting from lactose by regrouping the glucose residue to the fructose molecule with a passage form an aldose form to ketosis one (Aidera and de Halleux, 2007). It is not digested in the upper gastrointestinal tract of human but is fermented by colonic bacteria. The bifidogenic nature of lactulose at doses of 10 g/day has been confirmed, using both traditional microbiological culture techniques and fluorescent *in*

situ hybridization with molecular probes for bacterial enumeration, in a double-blind placebo-controlled study (Kolida *et al.*, 2000). Infants fed formula supplemented with a mixture of polydextrose (PDX), galactooligosaccharides (GOS), and lactulose (LOS), achieved normal growth and stool characteristics more similar to those of breast-fed infants in comparison with infants fed an unsupplemented formula (Ziegler *et al.*, 2007).

Galactooligosaccharides (GOS) are nondigestible carbohydrates which are resistant to gastrointestinal digestive enzymes, present in human milk, cows' milk and yogurt and may also be producted synthetically from lactose, but are fermented by specific colonic bacteria (Kolida *et al.*, 2000; Sako *et al.*, 1999). GOS are produced from lactose by the action of β- galactosidases which have transgalactosylation activity (Mussatto and Mancilha, 2007; Sako *et al.*, 1999). The linkage between the galactose units, the efficiency of transgalactosylation, and the components in the final products depend on the enzymes and the conditions used in the reaction (Sako *et al.*, 1999). Alliet *et al*. (2007) studied cholesterol and triacylglycerol levels in infants receiving a formula with a specific mixture of 0.6 g 100 ml^{-1} of galactooligosaccharides (GOS) and long chain fructooligosaccharides (FOS) in a ratio of 9:1, a control formula, or breast milk. Total cholesterol and LDL levels at 8 and 26 week were significantly lower in the formula-fed groups than in the breast-fed infants.

Fructooligosaccharides (FOS) are polymers of D-fructose joined by β (2→1) bonds with an α (1→2) linked D-glucose at the terminal end of the molecule (Kolida *et al.*, 2000). Production can be divided into two classes: in the first one, they are produced from the disaccharide sucrose using the transfructosylation activity of the enzyme β-fructofuranosidase. The second method used for fuctooligosaccharides production is the controlled enzymatic hydrolysis of the polysaccharide inulin (inulin oligofructose), which can be extracted from chicory roots, onion, garlic, Jerusalem artichoke, tomato and banana (Kolida *et al.*, 2000; Mussatto and Mancilha, 2007). Bouhnik *et al*., (1999) demonstrated that short chain FOS administration dose-dependently increase faecal bifidobacteria in healthy humans. In rats fed a lipid-rich diet containing 100 g fructooligosaccharides kg^{-1}, a decrease in triacylglycerolaemia also occurs without any protective effect on hepatic triacylglycerol accumulation and lipogenesis, suggesting a possible peripheral mode of action (Delzenne, 2003).

Isomaltooligosaccharides (IMO) are derived from starch by a two-step enzymatic process and are mixtures of α (1→6) glucoside such as isomaltose, isomaltotriose, panose and isomaltotritraose. Isomaltooligosaccharides are potentially digested by isomaltase in the human jejunum and the residual oligosaccharides are fermented by bacteria in the colon. A number of studies have suggested that IMO are bifidogenic (Kolida *et al.*, 2000). They have been shown to be fermented by bifidobacteria and *Bacteriodes fragilis* but not by *Escherichia coli* and other bacterial population (Fooks *et al.*, 1999).

Xylooligosacharides (XOS) are polymer of D-xylose (Kolida *et al.*, 2000). Production of XOS at an industrial scale is carried out from the polysaccharide xylans extracted from lignocellulose materials. Typical raw materials for xylooligosaccharides production are hardwoods, corn cobs, straws, bagasses, hulls, malt cakes and bran. Three different approaches have been used for xylooligosaccharides production from these feedstocks: (a) Enzyme treatments of native xylan-containing lignocellulosic material; (b) Chemical fractionation of a suitable lignocellulosic material to isolate (or to solubilize) xylan, with further enzymatic hydrolysis of this polymer to xylooligosaccharides; and (c) Hydrolytic degradation of xylan to xylooligosaccharides using steam, water or dilute solutions of mineral acids (Mussatto and Mancilha, 2007). XOS be applied as prebiotic food ingredients, hence its positive health effect (Vázquez *et al.*, 2006). Marinho *et al*. (2007) evaluated the effect of a prebiotic or probiotic on the piglets intestinal fermentative activity. Piglets received one of the following diets: basal diet (C); basal diet supplemented with xylooligosaccharides (C-XOS); basal diet supplemented with *Saccharomyces cerevisiae* (C-SC); and basal diet supplemented with xylooligosaccharides and *S. cerevisiae* (C-XOS+SC). The xylanolytic activity was higher ($P<0.05$) in the small intestine of piglets fed C-XOS+SC diet, but no significant difference as found in the caecum and colon.

Soybean oligosaccharides refered to oligosaccharides found in soybeans are extracted directly from the raw material and do not require enzymatic manufacturing processes (Lana *et al.*, 2007; Mussatto and Mancilha, 2007). Soybean whey, a by-product from the production of soy protein isolates and concentrates, contains the oligosaccharides raffinose, stachyose, and verbascose, which consist of 1, 2, or 3 α (1→6) linked units of galactose linked through α (1→3) bonds to a terminal sucrose. The oligosaccharide found in the highest concentration is stachyose, followed by raffinose, followed by verbascose

(Mussatto and Mancilha, 2007). *In vivo* experimental results showed that dietary SMO increased visible microbial populations attached on caecal walls and increased the population of a group of lactic acid bacteria (genera of *Lactobacillus, Pediococcus, Weissella* and *Leuconostoc*) in the caecal contents of young broiler chickens ($P<0.05$). In conclusion, SMO does show promise for use as a product which may promote competitive exclusion of potential pathogens.

6. Synbiotics

A further possibility in microflora management procedures is the use of synbiotics, where probiotics and prebiotics are applied in combination (Fooks *et al.*, 1999). Recent research in the area of prebiotic oligosaccharides and synbiotic combinations with probiotics is leading towards a more targeted development of functional food ingredients. Improved molecular techniques for analysis of the gut microflora, new manufacturing biotechnologies, and increased understanding of the metabolism of oligosaccharides by probiotics are facilitating development. Such developments are leading us to the time when we will be able to rationally develop prebiotics and synbiotics for specific functional properties and health outcomes (Tzortzis *et al.*, 2004). Colonic microflora has a profound influence on health and prebiotic oligosaccharides as functional food ingredients to manipulate the composition of colonic microflora in order to improve health (Rastall and Maitin, 2002) therefore, consumption of a probiotic in combination with a suitable prebiotic (synbiotic) can result in synergistic effects, improving survival of the probiotic by providing a readily available substrate for fermentation and by increasing autochthonous bifidobacteria levels (Bartosch *et al.*, 2005). Geier *et al*. (2005) compared the effects of the prebiotic, fructooligosaccharides alone and in synbiotic combination with *Lactobacillus fermentum* BR11 on the development of dextran sulfate sodium-induced colitis in rats. No protection from dextran sulfate sodium-colitis was accorded by fructooligosaccharide alone or in synbiotic combination with *L. fermentum* BR11. Bartosch *et al*. (2005) studied microbiological effects of consumption of a synbiotic containing *Bifidobacterium bifidum, Bifidobacterium lactis,* and oligofructose in elderly persons. Viable bacteria were determined and counted by real time polymerase chain reaction. Throughout feeding, both bifidobacteria species were detected in fecal samples obtained from all subjects receiving the synbiotic with significant increases in the number of the 16S rRNA genes of *B. bifidum,*

B. lactis, and total bifidobacteria. Amerkhanova *et al*. (2006) evaluated the biologically active food supplement "Normospectrum" vs. the commercial preparation "Bifidumbacterin", its ability to correct the intestinal microflora and the functional condition of the gastrointestinal tract in children. "Normospectrum" was well tolerated, favored regression of the main clinical manifestations, and had positive effect on the intestinal microbiocenosis, increasing the proportion of bifidobacteria, lactobacilli and *Escherichia* with full enzymatic activity, and lowering the proportion of conditionally pathogenic bacteria and fungi in the intestinal tract. Furrie *et al*. (2005) developed synbiotic for use in Ulcerative colitis (UC) patients combining a probiotic, *Bifidobacterium longum*, and a prebiotic (Synergy 1), a preferential inulinoligofructose. Short term synbiotic treatment of active UC resulted in improvement of the full clinical appearance of chronic inflammation in patients receiving this therapy.

Research objectives

1. To isolate probiotic bacteria from infant feces.
2. To screen and identify probiotic bacteria from infant feces.
3. To determine antibiotic susceptibility of selected lactic acid bacteria and bifido-bacteria strains isolated from healthy infant feces.
4. To examine the adhesion ability of probiotic lactobacilli and bifidobacteria isolated from infant feces to mucin.
5. To screen plant prebiotic extracts enhancing growth of the selected probiotic bacteria.
6. To develop of synbiotic product and probiotic viability during storage.
7. To investigate the effect of synbiotic supplement on the dominant human gut microflora and growth of S*almonella enterica* subsp. *enterica* serovar Typhimurium SA 2093 in challenging system under simulated human proximal colon conditions.

CHAPTER 2

ISOLATION AND SCREENING OF PROBIOTIC BACTERIA FROM INFANT FECES

2.1 Abstract

Lactic acid bacteria (LAB) and bifidobacteria were isolated from 25 healthy infant feces. The 313 strains of LAB and 17 strains of bifidobacteria were obtained. Abundances of LAB was greater found in 2-5 months old than the one from 2-6 days old infants. Whereas, isolated bifidobacteria were obtained only from feces of 2-6 days old infant. The number and colonization rate of LAB and bifidobacteria in natural born infants were higher than in cesarean born infant. The isolated LAB and bifidobacteria were screened for their probiotic properties including an ability to withstand a low pH and high bile concentration and antimicrobial activity. Fifty strains of LAB and 6 strains of bifidobacteria were able to grow in MRS agar containing 0.3% ox-gall bile with a survival rate higher than 80%. After, passing through acidified 0.85% NaCl (pH 2) containing 3g l^{-1} pepsin, the 11 strains of LAB and 5 strains of bifidobacteria were able to withstand this condition with survival rate higher than 80%. The survival of these bacteria ranged from 80.2-92.3% and 86.97-95.84%, respectively. The ten strains out of eleven strains of LAB and all 5 strains of bifidobacteria were able to maintain their viability after passing through gastrointestinal transit condition (acidic condition for 3 h followed by high bile salt for 6 h). Among the survival LAB only 2 strains of NIF1A7 and NIF1AN12 (identified as *Lactobacillus casei*) and 5 strains of CIF17A2, CIF17A4, CIF17A5, CIF17AN2 and CIF17AN8 (identified as *Lactobacills plantarum*) showed strong survival rate (>70%). Whereas survival rate of all 5 bifidobacteria identified as *Bifidobacterium longum* subsp. *longum* (NIF3AN3 and NIF7AN2) and *Bifidobacterium bifidum* (NIF7AN3, NIF7AN5 and NIF7AN10) was reached to 90%. In addition, acid and bile salt tolerance LAB and bifidobacteria also produced organic acid to inhibit the growth of both Gram-positive and Gram-negative pathogenic bacteria with inhibition activity ranging from 10 to 80 AU ml^{-1}. Only two strains of *L. plantarum* (CIF17AN2 and CIF17AN8) produced both organic acid and H_2O_2 to antagonize pathogenic bacteria. The assay used in this study allows an initial

assessment of strains for used as probiotic prior to select potential candidate strain for further study.

2.2 Introduction

Probiotics are defined as living microorganisms that confer various health benefits and promote or support a beneficial balance of the autochthonous microbial population of the gastrointestinal tract (Holzapfel and Schillinger, 2002)**.** Most probiotic organisms are lactobacilli or bifidobacteria, which are normal inhabitants of the human gut. Probiotic bacteria in sufficient numbers approximately 10^6-10^7 cell g^{-1} of food (Collado and Sanz, 2006; Dave and Shah, 1997; Lourens-Hattingh and Viljoen, 2001) exhibited a variety of health benefit through various mechanisms. The efficacy includes provision of essential vitamins (Wang *et al.,* 2010a); antimicrobial activity (Lee *et al*., 2003; Lin *et al.,* 2009; Tsai *et al*., 2008); anticarcinogenic activity (Burns and Rowland, 2000; Liong, 2008); improvement of immune response (Christensen *et al*., 2002); reduction of serum cholesterol (Jones *et al.,* 2011; Walker and Gilliland, 1993); alleviation of inflammatory bowel diseases (Sator, 2004) and increase of resistance to infectious diseases in gastrointestinal tract, (Resta-Lenert and Barrett, 2003) etc. Numerous health benefits of many strains of *Lactobacillus acidophilus, Lactobacillus paracasei* and *Bifidobacterium* originated from both humans and animals have been extensively investigated both *in vitro* and *in vivo*. These 2 genera are classified as Generally Recognized as Safe (GRAS) by US Food and Drug Administration because of their long history of safe use and their natural inhabitant in human intestine. Beside human commensal *Lactobacillus* and *Bifidobacterium* are thought to be better in adaptation to survive in highly stressful environment of gastrointestinal tracts and can adhere to the human epithelial wall of intestine much better than those from other sources. Therefore they are most extensively studied probiotics and preferentially used as commercial probiotics (Pinto *et al.,* 2006).

Several probiotic bacteria now being exploited commercially are mostly isolated from humans. For example, *Lactobacillus casei* Shirota, *Lactobacillus rhamnosus* GG (ATCC 53103) was from human intestine (Silva *et al*., 1987; Wills, 2012). *Lactobacillus acidophilus* LA-1 and *Lactobacillus reuteri* strains mm4-1a (ATCC PTA 6475) were isolated from human breast milk and human oral cavity (Oh *et al.,* 2009), respectively. Borriello *et al*. (2003) suggested that the ideal bacteria applied for

development of novel probiotics should be isolated from human fecal microflora of healthy volunteers.

LAB isolated from the feces of newborn babies is often able to survive a low pH and a relatively high bile salt concentration with little or no decrease in viable cell population (Park *et al.,* 2002). *Lactobacillus* spp. isolated from the feces of breast-fed infants have also shown resistance to gastric juice and bile salts and the ability to adhere to Caco-2 cells and to elicit antibacterial activity against both Gram-positive and negative potential pathogens (Park *et al.,* 2002).

The objective of this study was to screen, identify and assess potential probiotic lactic acid bacteria and bifidobacteria isolated from the feces of Thai infant by evaluating their acid resistance, bile resistance, antimicrobial activity n *in vitro*.

2.3 Materials and methods

2.3.1 Isolation of probiotic bacteria from infant feces

LAB and bifidobacteria were isolated from feces of 2-5 months and 2-6 days old; healthy breast-fed, natural-born and caesarean-born infants (Table 3). Fresh fecal samples were collected and kept in anaerobic jar and then transported to laboratory within 1 h. Bacterial isolation of ten-fold diluted fecal sample (in phosphate buffer containing 0.05% L-cysteine) was conducted rapidly by plating with modified de Man Rogosa agar (MRS) containing 0.05% L-cysteine and 0.004% bromocresol purple (Hoddadin *et al.,* 2004). Plates were incubated at 37°C for 48-72 h under aerobic and anaerobic conditions in an anaerobic jar with disposable BBL gas pack (Toure *et al*., 2003; Vlkova *et al*., 2005). Presumptive colonies of lactic acid bacteria (acid producing colony) were picked from the plate based on the variable colony characteristics. Each single colony was repeatedly transferred by streaking on the freshly prepared MRS agar to obtain a pure bacterial culture. The bacterial isolates were preliminary screened based on their morphological characteristic, Gram staining and catalase test results (Chung *et al*., 1999; Lievin *et al.,* 2000). Colonies exhibiting the presumptive characteristics of LAB (rod or short rod or cocci) and bifidobacteria (bifid) with Gram-positive and catalase negative were subcultured in L-cysteine containing MRS broth and stored in 30% glycerol in liquid nitrogen (-196°C).

2.3.2 Strains and culture condition

LAB was cultivated routinely in MRS broth (Himedia, Mumbai, India). Bifidobacteria were cultured in the modified MRS broth containing 0.05% L-cysteine and 1 mg l^{-1} resazurin in injection vial, which was sealed with rubber stopper and secured by aluminium cap, after being filled with nitrogen gas to achieve anaerobic condition and incubated at 37°C for 24-48 h. *Escherichia coli* TISTR 780 and *Staphylococcus aureus* TISTR 1466 were from Microbiological Resources Centre (MIRCEN), Thailand. *Escherichia coli* O157:H7 DMST 12743 was from the Department of Medical Science, Ministry of Public Health (Bangkok, Thailand). All patient isolates of *Listeria monocytogenes*, *Shigella sonei*, *Shigella flexneri*, *Salmonella enterica* subsp. *enterica* serovar Typhimurium SA2093 and *Salmonella* Paratyphi A were obtained from Microbiological Laboratory of Songklanakarind Hospital, Prince of Songkla University, Hat Yai (Songkhla, Thailand). These pathogens were cultivated in Muller Hinton broth (MHB; Himedia, Mumbai, India) at 37°C for 24 h.

2.3.3 Ability to grow in medium containing bile salt

LAB strains (313 strains) were cultured in MRS broth. Bifidobacteria (17 strains) were cultured in injection vial filled with MRS broth containing 0.05% L-cysteine, then sealed with rubber stopper and secured by aluminium cap, after being filled with nitrogen gas to achieve anaerobic condition. The tested tubes were incubated at 37 °C for 24-48 h, and 10% of which was transferred to fresh medium. After incubation the isolated LAB and bifidobacteria were tested for growth in MRS agar containing 0 and 0.3% ox-gall bile. One milliliter of cell culture was plated onto sterilized plate and pour plate with MRS agar with and without 0.3% (w/v) bile salt. Plates were incubated at 37°C for 24-72 h. The bacterial counts in the presence and absence of bile salt were expressed as log mean colony forming units (CFU) per ml of the original volume of the culture broth. The ability of the bacteria to grow in 0.3% bile salt was expressed as % survival, which was calculated using the equation below:

$$\% \text{ Bile survival} = (\log N_1 / \log N_0) \times 100$$

$\log N_1$ = Viable count (log CFU/ml) from 0.3% bile salt containing MRS agar

$\log N_0$ = Viable count (log CFU/ml) from MRS agar without bile salt

2.3.4 Low pH tolerance

Overnight cultures of LAB (50 strains) and bifidobacteria (8 strains) expressed bile salt tolerance were inoculated (10% v/v) into MRS broth and incubated at 37 °C for 24-48 h. One milliliter of the culture broth was taken and centrifuged at 4 °C, 10,000 rpm for 10 minute. The cell pellet was resuspended in 1 ml of gastric juice (0.85% NaCl containing 3 g l^{-1} pepsin adjusted to pH 2 with HCl) (Frece *et al*., 2005). The mixture was incubated at 37°C for 3 h with gentle agitation (Khalil *et al*., 2007). Total viable counts were performed before (log N_0) and after (log N_1) acid exposure by pour plate method using MRS agar for LAB and directly counted by using Live/Dead BaclightTH Bacterial viability kits according to manufacturer's recommendation (Eugene, Oregon, USA) for bifidobacteria. Green fluorescence of live cells and red fluorescence of dead cells were observed by using fluorescence microscope. The results were expressed as percentage of survival calculated from the following equation below. The isolates showing survivability more than 80% were selected for further studies.

$$\% \text{ Survival} = (\log N_1 / \log N_0) \times 100$$

2.3.5 Resistance to simulated gastrointestinal transit

Cell culture of LAB (11 strains) and bifidobacteria (5 strains) were centrifuged. Cell pellet was firstly exposed to acidic condition (0.85% NaCl containing 3 g l^{-1} pepsin and adjusted to pH 2 with HCl). The test tubes were incubated at 37 °C for 3 h with gentle agitation on a shaker. After that acid treated LAB and bifidobacteria were centrifuged and resuspended with bile salt solution (0.3% ox-gall bile and 3 mg ml^{-1} pancreatin). The test tubes were incubated at 37 °C for 6 h with gentle agitation. Total viable counts were performed before (log N_0) and after (log N_1) simulated gastrointestinal transit exposure as describe in 2.3.4.

2.3.6 Antimicrobial activity

Antimicrobial activity of 11 strains of LAB and 5 strains of bifidobacteria against various foodborne pathogens was performed using a broth microdilution assay in 96-well plates. Cell-free culture supernatant (CFCS) of LAB grown in MRS broth at 37°C for 24 h was obtained by centrifuging at 10,000 rpm at 4 °C for 10 min. One part of the supernatant was adjusted to pH 6-7 with 1 N NaOH, and then sterilized by filtration

through a 0.22 μm pore-size nitrocellulose membrane (Brink *et al*., 2006). In order to determine the effect of hydrogen peroxide involvement on antimicrobial activity, the neutralized supernatant (pH 6-7) was treated with catalase enzyme (final concentration of 200 unit mg^{-1}) at 25 °C for 30 minute and filtered (Moneke *et al*., 2009). A series of two-fold dilutions of the cell free culture supernatant (100 μl) in Muller Hinton broth (MHB) (100 μl) were prepared in triplicate in a 96-well microtiter plate. Bacterial pathogens including *E. coli* O157:H7, *E. coli* TISTR 780, *S. aureus* TISTR 1466, *S. sonnei, S. flexneri, Sal.* Paratyphi A, *Sal. enterica* subsp. *enterica* serovar Typhimurium SA2093 and *L. monocytogenes* were cultivated in MHB at 37°C for 24 h. The cultures were diluted to achieve 1-6 × 10^6 CFU ml^{-1} with MHB, respectively. Ten micro litter of each pathogen was then added to each well. Plates were incubated at 37°C for 24 h. The positive control contained undiluted supernatant and the negative control contained MHB plus pathogens. The inhibition activity was expressed as an arbitrary unit (AU) ml^{-1} calculated according to (1000/100) *D*, whereas *D* was the dilution factor (Millette *et al*., 2007).

2.3.7 Identification of LAB and bifidobacteria

2.3.7.1 DNA extraction

Genomic DNA was prepared from 1.5 ml of an overnight bacterial culture in MRS broth. Cells were harvested by centrifugation and resuspended in 560 μl TE buffer followed by adding 7μl lysozyme solution (50 mg ml^{-1}) and incubated at 50 °C for 1 h. Then 30 μl of 10% SDS and 3 μl of proteinase K were added. The suspension was incubated at 37 °C for 60 min. After the incubation, 267 μl of 5 M NaCl was added and mixed by repeated tube inversions then adding 80 μl of 10% CTAB in 0.7 M NaCl and further incubated at 65 °C for 10 min. DNA was extracted by adding an equal volume of phenol chloroform:isoamylalcohol (25: 24 : 1) followed by centrifuging at 10,000 rpm for 10 min. The DNA solution at the top layer was removed to a new tube and precipitated with isopropanol. The precipitated DNA was washed with 70% ethanol and centrifuged at 10,000 rpm for 10 min. The DNA was air dried and resuspended in 20-50 μl milli-Q water.

2.3.7.2 PCR conditions and analysis of PCR products

Amplification was carried out in the thermal cycler. The primer 27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492-r (5'-GGTTACCTTGTTACGACT T-3') were used to generate a 1,465 bp of product, corresponding to nucleotide 27-1492 of the 16S rRNA of LAB and Im26-f (5′GATTCTGGCTCAGGATGAACG-3′) and Im3-r (5′-CGGGTGCTICCCCACTT-TCATG-3′) were used for amplification 1,417 bp fragment of bifidobacterial 16S rRNA gene. The reaction mixture (50 µl) contained approximately 10 µM of each primer, 10× PCR buffer (Invitrogen, Brasil) 10 mM dNTP (Fermentas, Germany), 50 mM $MgCl_2$ (Invitrogen, Brasil), Taq DNA polymerase (Invitrogen, Brasil), 5 µl of bacterial DNA. DNA fragments of LAB were amplified as following condition: initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 2 min, annealing at 55 °C for 1 min, extension at 72 °C for 1.30 min and a 5-min final extension step at 72 °C. The following PCR program was used for bifidobacteria: initial denaturation at 94 °C for 5 min; 30 cycles of 94 °C for 30 s, 57 °C for 30 s, and 68 °C for 1 min 30 s; 57 °C for 30 s; and 68 °C for 7 min (Satokari *et al.*, 2001). Aliquots of the PCR products were subjected to electrophoresis in a 1% agarose gel in TAE buffer. Gels were stained with ethidium bromide and visualized under UV light. A 100 bp DNA ladder was used as a molecular marker. PCR products were purified by using the NucleoSpin® Extract II (Germany) according to the manufacturer's instructions.

2.3.7.3 DNA sequencing

The purified PCR products were sequenced by BioDesign Co.,Ltd., Thailand. The nucleotide sequences of the selected strains were compared with sequence data in nucleotide database deposited in GenBank, using the BLAST search program (available at http://www.ncbi.nlm.nih.gov/) with sequence data held at GenBank. The nucleotide sequences of the 16S rDNA of the potential probiotic bacteria and the nucleotide sequences of the reference strains were aligned with the Clustal X program (2.1.12). The phylogenetic tree was constructed using the Mega 5 program.

2.4 Results

2.4.1 Isolation and screening of probiotic bacteria from infant feces

Fecal samples were collected from infants born at Songklanakarin Hospital. The 330 strains of lactic acid bacteria (LAB) and bifidobacteria were isolated from 25 fecal samples (Table 3). After Gram staining and catalase test, 313 isolates were presumptively classified as LAB according to morphological characteristics (rod or short rod, Gram-positive and catalase negative), and 17 strains were bifidobacteria based on their bifid shape, Gram-positive and catalase negative characteristics. High numbers of presumptive LAB was isolated from feces of 2-5 months old, natural born and both breast and formula-fed infants (Table 4). Whereas, bifidobacteria was only found in first weak, natural born and both breast and formula-fed infant's feces. The number and colonization rate of LAB and bifidobacteria in natural born infants were higher than in cesarean born infant. Mean bacterial abundances of LAB and bifidobacteria of infant's feces was shown in Table 5. The highest number of LAB and bifidobacteria (9.63 log CFU g^{-1} feces) was counted from the feces of 2-6 days old, natural born, breast-fed infant whereas the lowest LAB and bifidobacter abundance 8.41 log CFU g^{-1} feces was found in 2-6 days old, cesarean born, both breast and formula-fed infants.

2.4.2 Survival of LAB and bifidobacteria in the upper part of gastrointestinal tract

2.4.2.1 Bile salt tolerance

The ability to grow in MRS agar containing bile salt revealed variation in species or strains of lactic acid bacteria and bifidobacteria isolated from the infants. Among 313 strains of LAB, 186 strains obtained from 18 fecal samples were able to grow medium containing 0.3% ox-gall bile (Table 6). While, loss of cell viability was found in 127 strains of LAB obtained from 7 fecal samples which aged less than one week old and cesarean born infant. Fifty strains out of 186 bile salt tolerance isolates showed survival higher than 80% with viable cell counts above 7 log CFU ml^{-1} on average. Among 17 obtained isolates of bifidobacteria, eight strains were able to grow in medium containing 0.3% ox-gall bile. Percentage of bile salt tolerance in these strains was ranging from 79.49% to 94.98%. Only

6 out of 8 bile salt tolerance bifidobacteria had the survival rate higher than 80% with viable cell ranging from 6.33 to 7.45 log CFU ml^{-1}. LAB and bifidobacteria with survival higher than 80% were selected for further study.

2.4.2.2 Acid tolerance

The effect of acidity on the viability of 50 strains LAB and 6 strains of bifidobacteria were assessed by adjusting pH of 0.85% NaCl containing 3 g l^{-1} pepsin to pH 2 and incubated at 37 °C for 3 h. Forty-seven strains out of 50 strains survived from high bile salt concentration were able to survive at pH 2 for 3 h. The eleven strains (CIF17A5, CIF1A10, CIF1A1, CIF1A2, CIF17AN2, NIF1A7, CIF17A2, NIF1AN12, CIF17A4, CIF17AN8, and CIF1A9) with high survival were selected with survival of 92.29, 91.85, 89.43, 88.72, 88.56, 86.34, 85.27, 84.08, 80.85, 80.37 and 80.18%, respectively (Figure 6). The selected strains were isolated from feces of more than 2 months old infant. The tolerant LAB under extreme conditions of gastrointestinal tract in this study was found with increasing the age of infants. The survival of bifidobacteria was shown in Figure 7. Only 5 strains of bifidobacteria had survival more than 80% with the viability between 6.19-7.51 log CFU ml^{-1}.

2.4.2.3 Resistance to the sequential exposure of gastric and bile of the selected LAB and bifidobacteria

The survival of the probiotics from the condition simulating gastrointestinal (GI) transit was presented in Table 7. The 11 strains of LAB and 5 strains of bifidobacteria were subjected to the sequential exposure of simulated gastric juice pH 2.0 for 3 h and simulated intestinal fluid containing 0.3% ox-gall bile for 6 h. All tested strains showed viability or ability to grow after exposure to simulated gastric condition for 3 h with survival rate higher than 80%.

Ten strains of LAB were able to withstand acid exposure followed by bile with survival rate ranging from 14.80 - 80.03%. Only strain CIF1A10 could not survive in the sequential exposure. However, 7 strains of NIF1A7, NIF1AN12, CIF17A2, CIF17A4, CIF17A5, CIF17AN2 and CIF17AN8 showed survival higher than 70% with log reduction of 1.84-2.52 log CFU ml^{-1}. The strain CIF17AN8 exhibited the highest survival of 80.03% with log reduction of 1.84 log CFU ml^{-1}.

All strains of bifidobacteria were still alive after passing through extreme conditions in stomach and small intestine. The survival of these strains was 86.97-95.84%.

Bifidobacteria NIF7AN2 was most tolerant strain with log reduction of 0.33 log cell ml^{-1}. Whereas, the strain NIF7AN5 was the most sensitive strain.

Table 3. Fecal samples.

No.	Fecal samples	Source of feces				Obtained isolates	
		mode of delivery	age	gender	type of feeding	LAB	Bifidobacteria
1	CIF1	cesarean born infant	4 months	male	breast and formula	20	-
2	CIF2	cesarean born infant	4 months	female	breast and formula	20	-
3	CIF3	cesarean born infant	4 months	female	breast and formula	19	-
4	CIF4	cesarean born infant	3 days	female	breast and formula	6	-
5	CIF5	cesarean born infant	2 days	male	breast and formula	23	-
6	CIF6	cesarean born infant	4 days	male	formula fed	17	-
7	CIF7	cesarean born infant	3 days	male	breast and formula	11	-
8	CIF8	cesarean born infant	3 days	male	breast and formula	15	-
9	CIF9	cesarean born infant	3 days	female	formula fed	8	-
10	CIF10	cesarean born infant	3 days	male	breast and formula	2	-
11	CIF11	cesarean born infant	4 days	female	breast and formula	5	-
12	CIF12	cesarean born infant	6 days	male	breast fed	10	-
13	CIF13	cesarean born infant	4 days	female	breast fed	10	-
14	CIF14	cesarean born infant	4 days	female	breast and formula	14	-
15	CIF15	cesarean born infant	5 days	male	breast and formula	4	-
16	CIF16	cesarean born infant	4 days	female	breast and formula	8	-
17	CIF17	cesarean born infant	5 months	male	breast and formula	17	-
18	NIF1	natural born infant	2 months	male	breast and formula	24	-
19	NIF2	natural born infant	2 days	female	breast fed	20	-
20	NIF3	natural born infant	3 days	male	breast and formula	12	4
21	NIF4	natural born infant	3 days	male	formula fed	10	-
22	NIF5	natural born infant	3 days	female	breast and formula	19	-
23	NIF6	natural born infant	3 days	female	breast and formula	12	-
24	NIF7	natural born infant	2 days	female	breast and formula	2	13
25	NIF8	natural born infant	4 days	male	formula fed	5	-
		Total				313	17

Table 4. Mean bacterial number isolated from infant feces.

Age	Sample	Mode of delivery	Sample	Bacteria	Numbers (mean)	Total
2-5 months	5	cesarean born delivery	4	LAB	55 (13.75)	79 (15.8)
				Bifidobacteria	0	
		natural born delivery	1	LAB	24	
				Bifidobacteria	0	
1 week	20	cesarean born delivery	13	LAB	137 (10.53)	251 (12.55)
				Bifidobacteria	0	
		natural born delivery	7	LAB	97(13.86)	
				Bifidobacteria	17 (2.42)	
	25		25			330

Table 5. Bacterial abundances of LAB and bifidobacteria in infant feces.

Ages	Mode of delivery	Type of feeding	Mean of log CFU g^{-1} (ranges)
5 months	cesarean born	breast and formula	9.33
4 months	cesarean born	breast and formula	9.44 (9.24-9.64)
2 months	natural born	breast and formula	9.28
2-6 days	cesarean born	breast and formula	8.41 (6.59-9.35)
2-6 days	cesarean born	breast	8.57 (8.19-8.94)
2-6 days	cesarean born	formula	9.17 (8.27-10.06)
2-6 days	natural born	breast and formula	8.82 (8.35-9.24)
2-6 days	natural born	breast	9.63
2-6 days	natural born	formula	8.73 (8.10-9.37)

Table 6. Number of bile tolerance LAB.

Fecal samples	Source of feces				Isolates obtained	Bile tolerant (% survival range)		Isolate number with > 80% survival
	Mode of delivery	Age	Gender	Type of feeding				
CIF1	cesarean born infant	4 months	male	breast and formula fed	20	9	(2.32-120%)	5
CIF2	cesarean born infant	4 months	female	breast and formula fed	20	13	(1.35-101.86%)	2
CIF3	cesarean born infant	4 months	female	breast and formula fed	19	16	(0.13-78.34%)	-
CIF4	cesarean born infant	3 days	female	breast and formula fed	6	6	(12.39-33.04%)	1
CIF5	cesarean born infant	2 days	male	breast and formula fed	23	23	(60.12-117.92%)	7
CIF6	cesarean born infant	4 days	male	formula fed	17	10	(41.02-111.65%)	2
CIF7	cesarean born infant	3 days	male	breast and formula fed	11	4	(14.29-68.71%)	-
CIF8	cesarean born infant	3 days	male	breast and formula fed	15	-	-	-
CIF9	cesarean born infant	3 days	female	formula fed	8	-	-	-
CIF10	cesarean born infant	3 days	male	breast and formula fed	2	-	-	-
CIF11	cesarean born infant	4 days	female	breast and formula fed	5	-	-	-
CIF12	cesarean born infant	6 days	male	breast fed	10	-	-	-
CIF13	cesarean born infant	4 days	female	breast fed	10	10	(54.08-119.70%)	7
CIF14	cesarean born infant	4 days	female	breast and formula fed	14	-	-	-
CIF15	cesarean born infant	5 days	male	breast and formula fed	4	4	(63.39-94.84%)	2
CIF16	cesarean born infant	4 days	female	breast and formula fed	8	-	-	-
CIF17	cesarean born infant	5 months	male	breast and formula fed	17	17	(71.78-107.08%)	9
NIF1	natural born infant	2 months	male	breast and formula fed	24	13	(1.03-103.18%)	2
NIF2	natural born infant	2 days	female	breast fed	20	16	(14.86-108.07)	6
NIF3	natural born infant	3 days	male	breast and formula fed	12	12	(52.97-97.22)	5
NIF4	natural born infant	3 days	male	formula fed	10	3	(66.68-94.39%	1
NIF5	natural born infant	3 days	female	breast and formula fed	19	11	(8.30-79.43%)	-
NIF6	natural born infant	3 days	female	breast and formula fed	12	12	(4.47-95.24%)	1
NIF7	natural born infant	2 days	female	breast and formula fed	2	2	(2.20-20.75%)	-
NIF8	natural born infant	4 days	male	formula fed	5	5	(3.37-12.47%)	-
	Total				313	186		50

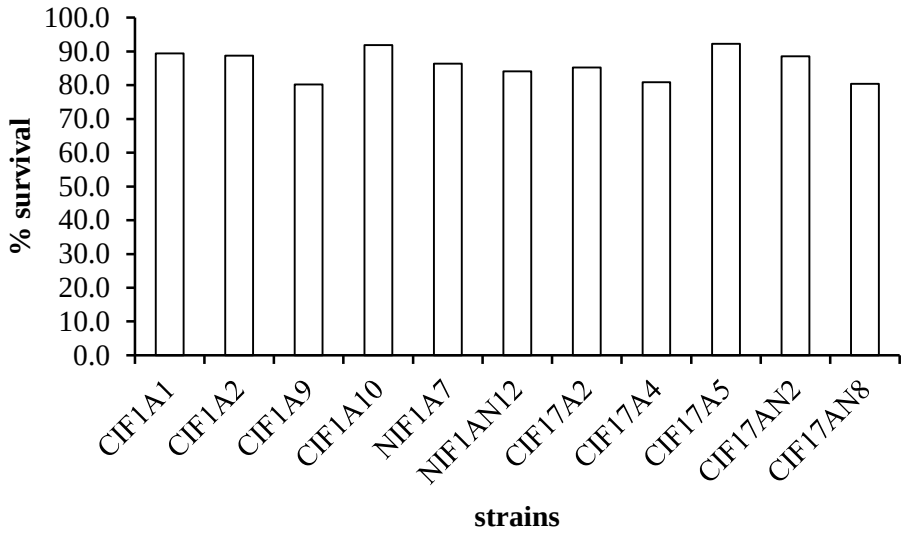


Figure 6. LAB survival after incubation in 0.85% NaCl containing 3 g l^{-1} pepsin adjusted to pH 2 at 37 °C for 3 h.

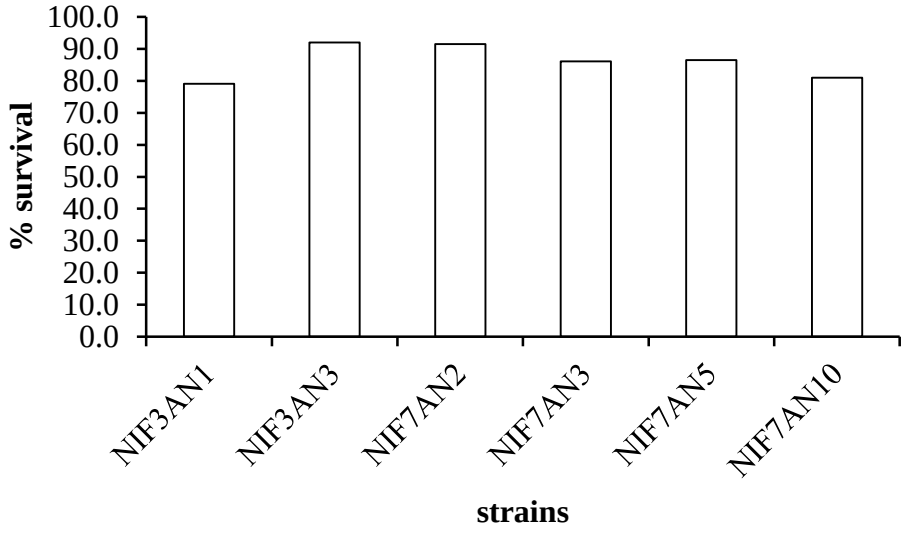


Figure 7. Bifidobacteria survival after incubation in 0.85% NaCl containing 3 g l^{-1} pepsin adjusted to pH 2 at 37 °C for 3 h.

Table 7. Survival of selected LAB and bifidobacteria after sequential incubation under the condition of the simulated gastrointestinal transit.

Strains	0 h (log CFU ml^{-1})	pH 2 (3h) (log CFU ml^{-1})	% survival	pH 2 (3h)+0.3% bile (6 h) (log CFU ml^{-1})	% survival
LAB					
CIF1A1	8.88±0.10	8.30±0.11	93.47	1.31±0.04	14.80
CIF1A2	8.73±0.08	8.58±0.19	98.33	1.61±0.03	18.39
CIF1A9	8.54±0.55	6.84±0.11	80.08	3.77±0.06	44.17
CIF1A10	8.78±0.10	8.42±0.07	95.91	-	-
NIF1A7	9.24±0.14	7.88±0.59	85.22	6.74±0.04	72.94
NIF1AN12	8.36±0.51	8.25±0.03	98.67	6.09±0.20	72.79
CIF17A2	9.19±0.01	8.18±0.14	89.03	6.86±0.18	74.62
CIF17A4	9.17±0.07	8.52±0.02	92.89	6.90±0.14	75.22
CIF17A5	9.29±0.08	8.10±0.03	87.26	6.77±0.39	72.85
CIF17AN2	9.17±0.12	8.31±0.06	90.54	7.14±0.08	77.87
CIF17AN8	9.21±0.03	8.31±0.02	90.27	7.37±0.02	80.03
Bifidobacteria					
NIF3AN3	7.68±0.05	7.29±0.07	94.98	6.79±0.09	88.45
NIF7AN2	8.01±0.01	7.45±0.03	93.04	7.68±0.20	95.84
NIF7AN3	7.63±0.013	6.67±0.028	87.36	7.09±0.11	92.94
NIF7AN5	8.14±0.03	6.60±0.05	81.15	7.08±0.09	86.97
NIF7AN10	7.70±0.12	6.33±0.25	82.27	6.89±0.16	89.52

2.4.3 Antimicrobial activity

Inhibitory against food-borne pathogens including *E. coli* TISTR, *S. aureus* TISTR 1466780, *E. coli* O157:H7, *L. monocytogenes*, *Sal.* Typhimurium SA2093, *Sal.* Paratyphi A, *S. sonneri* and *S. flexneri* and were determined using broth microdilution assay. Cell free culture supernatant (CFCS) of LAB (pH 3.9-4.06) and bifidobacteria (pH 4.25-4.65) exhibited antimicrobial activity against all tested indicator strains with the inhibition level from 10 AU ml^{-1} to 80 AU ml^{-1} (Table 8 and 9). However, the neutralized supernatant (NCFCS) (pH 6-7) from LAB strain CIF1A1, CIF1A2, CIF1A9, CIF1A10, NIF1A7, NIF1AN12, CIF17A2, CIF17A4 and CIF17A5 and all strains of bifidobacteria (NIF3AN3, NIF7AN2, NIF7AN3, NIF7AN5 and NIF7AN10) showed no inhibition against both Gram-positive and Gram- negative food-borne pathogens indicating

that the antimicrobial activities of these strains were affected by acid production. Only NCFCS of LAB strains CIF17AN2 and CIF17AN8 showed inhibitory against all tested strains except *Sal.* Typimurium SA 2093. However, loss of their antimicrobial activity was found after treating with 200 unit mg^{-1} catalase enzyme.

2.4.4 Probiotic identification and phylogenetic analysis

Table 10 showed the identification results of 16 probiotic bacteria strains. The nucleotide sequences of 16S rDNA of strains CIF1A1 showed 99% similarity with *L. rhamnosus* JCM^T1136 (D16552) type strain whereas strain CIF1A2, CIF1A9 and CIF1A10 showed 99% similarity to strain *L. rhamnosus* ATCC8530 (CP003094). The strains NIF1A7 and NIF1AN2 were similar to *L. casei* LC2W (CP002616) with similarity of 99%. The strains CIF17A2, CIF17A4 and CIF17A5 were identified as *L. plantarum* with 99% similarity with *Lactobacillus plantarum* WCFS (AL935263) and the strains CIF17AN2 and CIF17AN8 were identified as *L. plantarum* WCFS1with 99% similarity. The strain NIF3AN3 and NIF7AN2 were closely related to *B. longum* subsp. *longum* $JCM1217^T$ type strain with 99% similarity. The NIF7AN3, NIF7AN5 and NIF7AN19 strains were identified as *B. bifidum* with 99% similarity with *B. bifidum* YIT4039.

The phylogenetic tree of the 16S rRNA gene from 11 strains potential LAB (Figure 8) and 5 strains of potential bifidobacteria (Figure 9) was reconstructed using neighbor-joining method. *Vibrio vulnificus* $ATCC27562^T$ (X76333) and *Vibrio owensii* $DY05^T$ (GU018180) were used as out group. The bootstrap values above 50% were shown (based on 1,000 replications). The tree of potential LAB can be divided into 2 clades. The first clade consisted of *L. casei* and *L. rhamnosus*. The *lactobacillus* species in this group had high homology with each other and type strain. Another clade consists of only *L. plantarum* with also had high homology with each other and type strain. From this result indicated that *L. rhamnosus* were more closely related to *L. casei* than *L. plantarum*. The phylogenetic tree of potential bifidobacteria could be divided into 2 clades. The first clade consisted of the species of *B. longum* subsp. *longum* and another clade consisted of bacteria in species of *B. bifidum*. The bacterial strains in each group had high homology with each other.

Table 8. Antimicrobial activity of the selected lactic acid bacteria against food-borne pathogens by broth microdilution assay.

Test strains	Supernatant	Inhibition activity (AU ml^{-1})							
		E. coli TISTR 780	*S. aureus* TISTR 1466	*E. coli* O157:H7	*L. monocytogenes*	*Sal*. Typhimurium SA2093	*Sal*. Paratyphi A	*S. sonnei*	*S. flexneri*
CIF1A1	CFCS(pH4.04)	40	40	40	40	40	40	40	40
	NCFCS (pH 6-7)	-	-	-	-	-	-	-	-
CIF1A2	CFCS (pH4.05)	40	40	40	40	40	40	40	40
	NCFCS (pH 6-7)	-	-	-	-	-	-	-	-
CIF1A9	CFCS (pH4.06)	40	40	20	20	20	20	20	20
	NCFCS (pH 6-7)	-	-	-	-	-	-	-	-
CIF1A10	CFCS (pH3.9)	80	80	40	40	40	40	40	40
	NCFCS (pH 6-7)	-	-	-	-	-		-	-
NIF1A7	CFCS (pH3.9)	20	20	20	20	20	20	20	20
	NCFCS (pH 6-7)	-	-	-	-	-	-	-	-
NIF1AN12	CFCS (pH4.04)	20	20	20	20	20	20	20	20
	NCFCS (pH 6-7)	-	-	-	-	-	-	-	-
CIF17A2	CFCS (pH3.9)	20	20	20	20	40	20	20	20
	NCFCS (pH 6-7)	-	-	-	-	-	-	-	-
CIF17A4	CFCS (pH3.9)	20	20	20	20	40	20	20	20
	NCFCS (pH 6-7)	-	-	-	-	-	-	-	-
CIF17A5	CFCS (pH3.9)	20	20	20	20	40	20	20	20
	NCFCS (pH 6-7)	-	-	-	-	-	-	-	-
CIF17AN2	CFCS (pH3.9)	20	20	40	40	40	40	40	40
	NCFCS (pH 6-7)	20	20	10	10	-	10	10	10
CIF17AN8	CFCS (pH3.9)	20	20	40	40	40	40	40	40
	NCFCS (pH 6-7)	10	20	10	10	-	10	10	10

- No antimicrobial activity; CFCS = cell-free culture supernatant; NCFCS = neutralized cell-free culture supernatant

Table 9. Antimicrobial activity of the selected bifidobacteria against food-borne pathogens by broth microdilution assay.

Test strains	Supernatant	Inhibition activity (AU ml^{-1})							
		E. coli TISTR 780	*S. aureus* TISTR 1466	*E. coli* O157:H7	*L. monocytogenes*	*Sal*. Typhimurium SA2093	*Sal*. Paratyphi A	*S. sonnei*	*S. flexneri*
NIF3AN3	CFCS(pH4.25)	20	20	20	20	80	20	20	20
	NCFCS (pH 6-7)	-	-	-	-	-	-	-	-
NIF7AN1	CFCS (pH4.41	10	10	10	10	80	10	10	10
	NCFCS (pH 6-7)	-	-	-	-	-	-	-	-
NIF7AN2	CFCS (pH4.62)	10	10	10	10	80	10	10	10
	NCFCS (pH 6-7)	-	-	-	-	-	-	-	-
NIF7AN5	CFCS (pH4.65)	10	10	10	10	80	10	10	10
	NCFCS (pH 6-7)	-	-	-	-	-	-	-	-
NIF7AN10	CFCS (pH4.25)	20	20	20	20	80	20	20	20
	NCFCS (pH 6-7)	-	-	-	-	-		-	-

- No antimicrobial activity; CFCS = cell-free culture supernatant; NCFCS = neutralized cell-free culture supernatant

Table 10. Identification of probiotic lactic acid bacteria and bifidobacteria isolated from healthy infant feces.

Strains	Closest species in NCBI	Sequence accession number	Similarity (%)
CIF1A1	*Lactobacillus rhamnosus* JCM1136^{T}	D16552	99
CIF1A2	*Lactobacillus rhamnosus* ATCC8530	CP003094	99
CIF1A9	*Lactobacillus rhamnosus* ATCC8530	CP003094	99
CIF1A10	*Lactobacillus rhamnosus* ATCC8530	CP003094	99
NIF1A7	*Lactobacillus casei* LC2W	CP002616	99
NIF1AN12	*Lactobacillus casei* LC2W	CP002616	99
CIF17A2	*Lactobacillus plantarum* WCFS1	AL935263	99
CIF17A4	*Lactobacillus plantarum* WCFS1	AL935263	99
CIF17A5	*Lactobacillus plantarum* WCFS1	AL935263	99
CIF17AN2	*Lactobacillus plantarum* WCFS1	AL935263	99
CIF17AN8	*Lactobacillus plantarum* WCFS1	AL935263	99
NIF3AN3	*Bifidobacterium longum* subsp. *longum* JCM 1217^{T}	AP010888	99
NIF7AN2	*Bifidobacterium longum* subsp. *longum* JCM 1217^{T}	AP010888	99
NIF7AN3	*Bifidobacterium bifidum* YIT 4039	AB437356	99
NIF7AN5	*Bifidobacterium bifidum* YIT 4039	AB437356	99
NIF7AN10	*Bifidobacterium bifidum* YIT 4039	AB437356	99

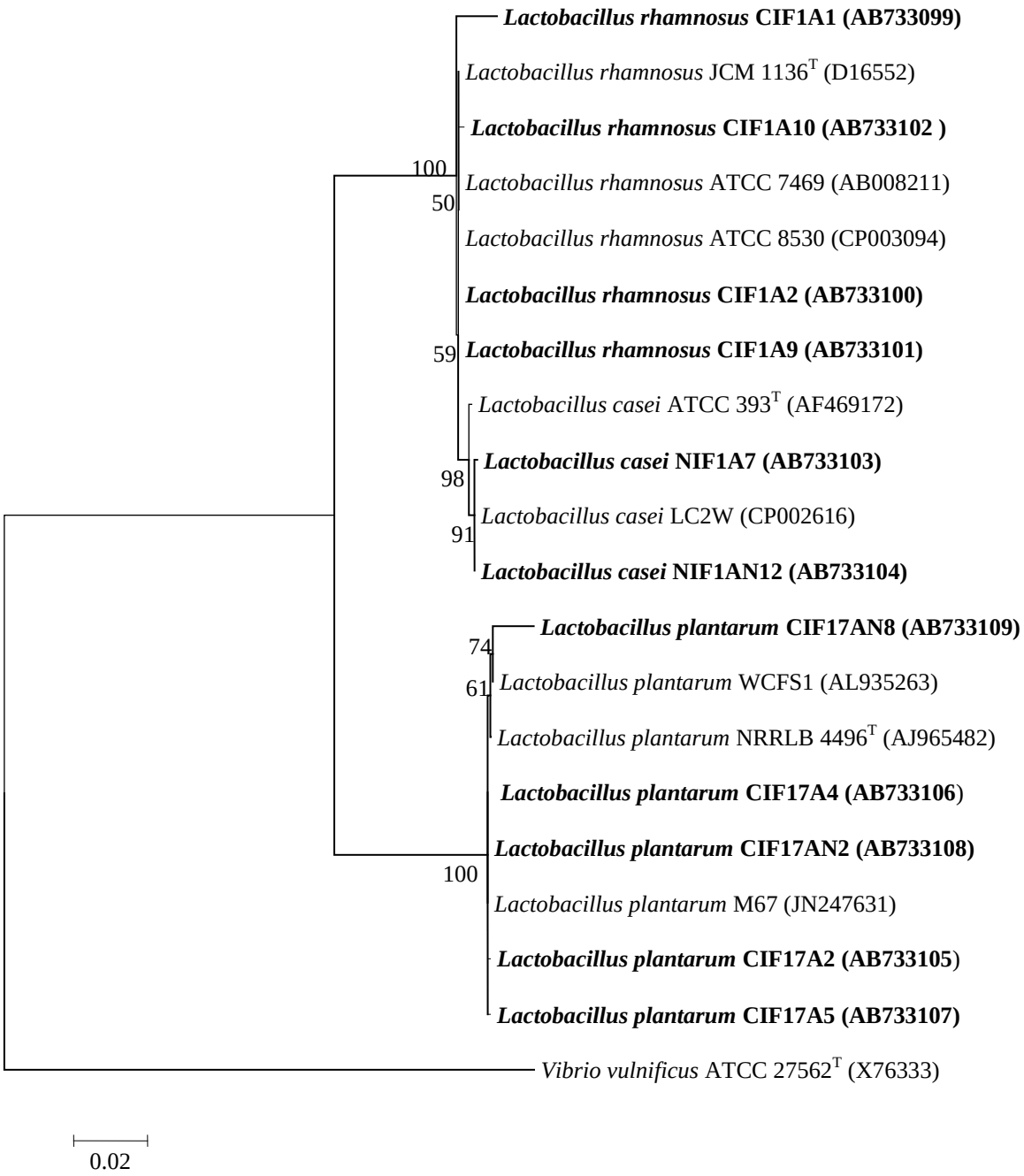


Figure 8. Phylogenetic tree based on the nucleotide sequences of 16S rRNA genes of 11 lactobacilli isolated from infant feces using neighbor-joining method. Bootstrap values for a total of 1,000 replicates were given. *Vibrio vulnificus* ATCC 27562^{T} was used as out group.

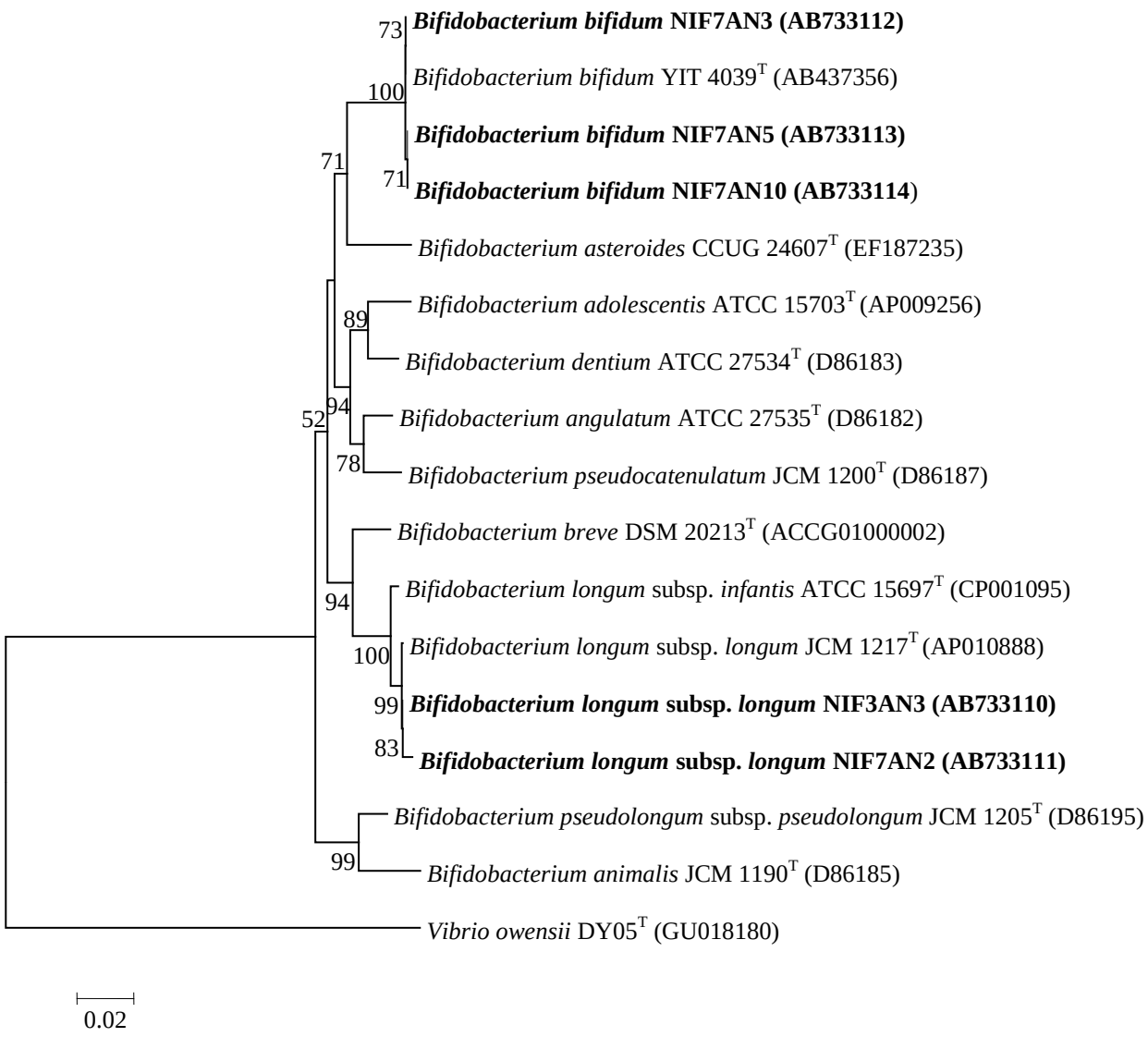


Figure 9. Phylogenetic tree based on the nucleotide sequences of 16S rRNA genes of 5 bifidobacteria isolated from infant feces using neighbor-joining method. Bootstrap values for a total of 1,000 replicates were given. *Vibrio owensii* DY05^{T} was used as out group.

2.5 Discussion

Feces of healthy infants were used as a source for isolation LAB and bifidobacteria. Lack of equipment for providing anaerobic condition during procedure and uncultured bifidobacteria resulted to low number of obtained bifidobacteria in this study. Only 17 strains of bifidobacteria were obtained in this study. Nearly all of them derived from feces of natural born, both formula and breast-fed infants. In full-term infants, a diet of breast milk induces the development of flora rich in *Bifidobacterium* spp. (Fanaro *et al.*, 2003). The colonization rate of *Bifidobacterium*-like bacteria and *lactobacilli*-like bacteria were consistently lower in cesarean babies compared to the vaginally delivered infants (Gronlund *et al*., 1999).

The abundance of LAB was also found in fecal sample of natural born, breast-fed infant. There are many factors influencing the development of infant gut microflora such as gestational age, mode of delivery, local environment, type of feeding, and antibiotic treatment (Westerbeek *et al*., 2006). During the first week of life, the initial colonizers were enterobacteria and streptococci in both formula and breast-fed babies, reaching their highest number by age 3.3 d on average. The bifidobacteria and bacteroides appeared 1 day later, and then reached their highest numbers on day 5-6 (Mackie *et al*., 1999). It was well established that the type of birth delivery had a significant effect on the development of the intestinal microbiota. Also, immediately after birth, the nasopharynx of 62% of babies contained bacteria that were consistent with those of their mothers' vaginas (Mackie *et al*., 1999). Cesarean born infant can also be exposed to their mothers' microbiota, but initial exposure was most likely to environmental isolates from equipment, air and nursing staff serving as vectors for transfer (Mackie *et al*., 1999). However, vaginal microbes usually do not settle in the intestinal tract. The maternal intestinal flora was on the contrary, a well-recognized source of bacteria for the newborn (Fanaro *et al*., 2003). Breast-feeding tends to contribute to higher levels of bifidobacteria, although with modern infant formula the differences were now less pronounced than in the past.

In order to exert positive health effects, the lactobacilli and bifidobacteria should resist the stressful condition of the stomach and upper intestine that contain bile (Pan *et al.*, 2009a). Acidity is believed to be the most detrimental factor affecting growth and viability of lactobacilli and bifidobacteria, because their growth was down significantly

below pH 4.5 (Pan *et al.,* 2009a). Bile salts are toxic for living cells, since they disorganize the structure of cell membrane (Guo *et al*., 2009). Our results found that LAB and bifidobacteria isolated from healthy infant feces survived from condition mimicked upper part of human gastrointestinal tract. The ability of LAB and bifidobacteria to tolerate to extreme condition in stomach and upper small intestine was the primary prerequisite of probiotic bacteria to reach the target site and exert beneficial effect on host health (Dunne *et al*., 2009; Sheehan *et al*., 2007). However, the acid and bile tolerance varied greatly depending on the species and tested strains. Similar result was report by Lim *et al.* (2004) 94.9% in *Lactobacillus* (37/39 strains) and 60.5% (26/43 strains) in *Bifidobacterium* isolated from human intestine were tolerable to bile salt. The high survival (>80%) of *L. rhamnosus* HN001, *L. rhamnosus* HN 067, *L. acidophilus* and *B. lactis* HN019 after 3 h expose to pH 3 and bile concerntration of 1% w/v were reported (Prasad *et al.,* 1999). *Lactobacillus johnsonii* La1 was added at 7 log CFU ml^{-1} and about 6 log CFU^{-1} of this strain showed survival after the incubation (Yamano *et al*., 2006). In simulated GI transit (gastric juice at pH 3 for 3 h and intestinal juice at pH 8 for 12 h), there were no considerable difference between *L. acidophilus* NCFM (99.6%) and *L. rhamnosus* GG (99.2%), between *L. casei* Zhang (97.4%) and *Lactobacillus shirota* (97.6%), respectively (Guo *et al*., 2009). The acid tolerance of LAB has been linked to the induction of H^+-ATPase activity (Guo *et al*., 2009). When exposed to acidic conditions, bacteria try to maintain a pH homeostasis by discharging H^+ from the cell by H^+-ATPase (Masco *et al*., 2007). Bile salt hydrolase (BSH) production bacteria was found to hydrolyze bile salts, which decreases the solubility of bile salt, and thus weakening their detergent effect in order to protect against the toxicity of bile acids (Shah and Liong, 2006).

As potential probiotic bacteria, antimicrobial activity is one of important properties. The 12 probiotic candidate including 7 strains of LAB and 5 strains of bifidobacteria showed strong antimicrobial activity against both Gram-positive and Gram-negative pathogenic bacteria. Most of their activities were due to production of organic acid. Production of lactic acid, the major metabolite of LAB, is responsible for the associated decrease in pH, which may be sufficient to antagonize many microorganisms. The solubility of the non-dissociated form and the insolubility of the ionized acid form of organic acids within the cytoplasmic membrane caused acidification of cytoplasm and the collapse of the motive force, resulting in inhibition of nutrient transport (Goncalves *et al*.,

1997). Several *Lactobacillus* strains from infant feces also produced acid to inhibit the growth of enteropathogens (Tsai *et al*., 2008; Wang *et al*., 2010a). As well, bifidobacteria were found to exert strong inhibitory activity towards Gram-negative indicator bacteria, namely *Sal. enterica* serovar Typhimurium SL1344 and *E. coli* C1845 by lowering of the pH of the medium and producing of organic acids (Makras and Vuyst, 2006). The similar results were found in 21 strains of lactobacilli isolated from newborn infant feces. Most of the strains were found to produce inhibition zone against some pathogenic bacteria. When the pH value of the supernatant was adjusted to 6.5 and catalase was added, there was reduction of inhibition against pathogenic bacteria (Arici *et al*., 2004).

While antimicrobial activities of strains CIF17AN2 and CIF17AN8 were the result of both acid and H_2O_2 production. Hydrogen peroxide is produced by LAB in the presence of oxygen as a result of the action of flavoprotein oxidases or nicotinamide adenine dinucleotide (NADH) peroxidase (Ammor *et al.*, 2008). The antimicrobial effect of H_2O_2 may result from the oxidation of sulfhydryl groups causing denaturing of a number of enzymes, and from the peroxidation of membrane lipids thus increasing membrane permeability (Ammor *et al.*, 2008).

After that, seven potential LAB strains were identified as *L. casei* NIF1A7, *L. casei* NIF1AN12, *L. plantarum* CIF17A2, *L. plantarum* CIF17A4, *L. plantarum* CIF17A5, *L. plantarum* CIF17AN2 and *L. plantarum* CIF17AN8. Whereas, low potential probiotic LAB strains with low survival rate after sequential exposure to gastric acid and bile were identified as *L. rhamnosus* CIF1A1, *L. rhamnosus* CIF1A2, *L. rhamnosus* CIF1A9 and *L. rhamnosus* CIF1A10. The potential bifidobacteria were identified as *B. longum* subsp. *longum* NIF3AN3, *B. longum* subsp. *longum* NIF7AN2, *B. bifidum* NIF7AN3, *B. bifidum* NIF7AN5 and *B. bifidum* NIF7AN10.

2.6 Conclusion

The 330 strains of LAB and bifidobacteria were isolated from 25 fecal samples. High numbers of presumptive LAB were isolated from feces of 2-5 months old, natural born and both breast and formula-fed infants. The numbers and colonization rate of LAB and bifidobacteria found in natural born infants were higher than in cesarean born infants. There were two strains of NIF1A7 and NIF1AN12 (identified as *L. casei*); five strains of CIF17A2, CIF17A4, CIF17A5, CIF17AN2 and CIF17AN8 (identified as

L. plantarum); 2 strains of NIF3AN3 and NIF7AN2 (identified as *B. longum* subsp. *longum*); 3 strains of NIF7AN3, NIF7AN5 and NIF7AN10 (identified as *B. bifidum*) showed strong survival in sequential exposure to simulated gastrointestinal (GI) transit. The cell free culture supernatant obtained from these bacteria showed inhibition against many foodborne pathogens including *E. coli* O157:H7, *E. coli* TISTR 780, *S. aureus* TISTR 1466, *Shi. sonnei, Shi. flexneri, Sal.* Paratyphi A, *Sal*. Typhimuruim SA2093 and *L. monocytogenes*. After partial characterization, we found that the antimicrobial activity of these bacteria was almost from organic acid. However, there was only the strains CIF17AN2 and CIF17AN8 (identified as *L. plantarum*) produced both organic acid and H_2O_2 to inhibit the tested pathogenic bacteria.

CHAPTER 3

ANTIMICROBIAL SUSCEPTIBILITY OF LACTIC ACID BACTERIA AND BIFIDOBACTERIA FROM INFANT FECES

3.1 Abstract

Eleven strains of lactic acid bacteria (LAB) belonging to *Lactobacillus rhamnosus* (CIF1A1, CIF1A2, CIF1A9 and CIF1A10) *Lactobacillus casei* (NIF1A7 and NIF1AN12) and *Lactobacillus plantarum* (CIF17A2, CIF17A4, CIF17A5, CIF17AN2 and CIF17AN8) and 5 strains of bifidobacteria belonging to *Bifidobacterium longum* subsp. *longum* (NIF3AN3 and NIF7AN2) and *Bifidobacterium bifidum* (NIF7AN3, NIF7AN5 and NIF7AN10) were tested against 9 antibiotic by broth microdilution assay in 96 well plates. All strains of LAB and bifidobacteria were susceptible to ampicillin, erythromycin, tetracycline and rifampicin. As well, all strains of tested bifidobacteria were sensitive to vacomycin, streptomycin and chloramphenicol. Polymyxin B resistance was detected in all tested strains except *B. longum* subsp. *longum* NIF3AN3. Similarly, vancomycin resistance was reported in all tested lactobacilli whereas, none of the tested bifidobacteria was resistant to this antimicrobial agent. However, resistant pattern of our human isolates lactobacilli and bifidobacteria to these 2 antimicrobial agents seemed to be intrinsically resistant. The minimum inhibitory concentration (MIC) value of chloramphenical toward *Lactobacillus rhamnosus* CIF1A9 (8 µg ml^{-1}) and *L. casei* NIF1A7 (8 µg ml^{-1}) showed just a bit above the breakpoint derived from the published body of research and from nation and European moitoring procedures (4 µg ml^{-1}). *Lactobacillus rhamnosus* CIF1A1, CIF1A9 and CIF1A10 were resistant to streptomycin with varying MIC values. Therefore, it is important to check the absence of transferable resistance gene in the last resistant group.

3.2 Introduction

The human gastrointestinal tract harbours a complex collection of microorganisms throughout its length, although it is the colon, which represents the main site of microbial colonization, providing residence for more than 500 different species of bacteria. LAB and bifidobacteria are indigenous members of the gastrointestinal microbiota of human. Bacterial strains intended to be used as probiotics in food systems which have to be systematically examined for antibiotic resistant determinants by the food chain. The presence of resistance genes in many LAB and the transfer of plasmids and conjugative transposons to and from LAB have been reported (Danielsen and Wind, 2003). This study was carried out to determine precisely the susceptibility of the selected LAB and bifidobacterial strains isolated from healthy infant feces, to 9 antimicrobial agents.

3.3 Materials and methods

3.3.1 Preparation of stock antibiotics

The safety of microorganisms used as probiotic strains is essential. Sensitivity of lactobacilli and bifidobacteria to different antibiotics was determined by broth microdilution assay (Toure *et al*., 2003; D'Aimmo *et al*., 2007; Domig *et al.*, 2007), and expressed in terms of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC).

Ampicillin, penicillin G sodium salt and vancomycin as inhibitors for cell wall synthesis; chloramphenicol sulfate, erythromycin, streptomycin sulfate, tetracycline hydrochloride as inhibitors for protein synthesis, polymyxin B sulfate as inhibitor of cytoplasmic function and rifampicin as nucleic acid synthesis inhibitor were used (Domig *et al*., 2007). Stock solutions (1 mg ml^{-1}) of ampicillin, penicillin G, vancomycin, streptomysin, polymyxin B and rifampicin were prepared by dissolving in water, whereas erythromycin, chloramphenicol and tetracycline were dissolved in 70% ethanol, then serial two-fold dilutions of 125 µl of the antibiotics were conducted in a 96-well polystyrene microplate containing 125 µl MRS broth per well to achieve final concentration raging from 256 to 0.125 µg ml^{-1}.

3.3.2 Determination of antibiotic susceptibility

Lactic acid bacteria (11 strains) were cultivated in MRS broth contained in screw cap tube and incubated at 37°C for 24 h and re-cultivated in the same medium. The cultures were adjusted to achieve $\approx 10^6$ CFU ml^{-1} with fresh MRS broth then 10 µl was added to microplate well contained varying concentration of different antibiotic to obtain final cell number of $\approx 10^4$ CFU ml^{-1}. The microplates were incubated at 37°C for 24-48 h.

Bifidobacteria (5 strains) were cultured in MRS broth containing 0.05% L-cysteine HCl, 1 mg l^{-1} resazurin contained in injection vial, which was sealed with rubber stopper and secured by aluminium cap, after being filled with nitrogen gas to achieve anaerobic condition and incubated at 37°C for 24-48 h and re-cultivted in the same medium. The cultures were adjusted to achieve $\approx 10^6$ CFU ml^{-1} with fresh MRS broth containing 0.05% L-cysteine then 10 µl was added to microplate well contained varying concentration of different antibiotic to obtain final cell number of $\approx 10^4$ CFU ml^{-1}. The microplates were placed in anaerobic jar with disposable BD gas pack and incubated at 37°C for 24-48 h. The MIC was defined as the lowest concentration of antibiotic giving a complete inhibition of visible growth in comparison to an antibiotic-free control well, while the MBC was the concentration that killed 99.9% of the initial inoculums. To determine mortality for the MBC determination, 10 µl was withdrawn from MIC and concentration higher than MIC to spread on MRS agar for determination of viable cell numbers (Rammanee and Hongpattarakere, 2011). MRS medium added with LAB, bifidobacteria or antibiotic was used as control. The resistance breakpoint was followed from Radulović *et al*. (2012) (derived from the published body of research and from nation and European moitoring procedures) as showed in Table 11. Bacteria with MIC values between these breakpoints are classified as intermediate. Isolates with MIC values above this breakpoint were classified as resistance. The isolates with MIC below the breakpoint were classified as susceptible.

3.4 Results

3.4.1 Antibiotic susceptibility of probiotics

The antimicrobial susceptibility toward 9 antibiotics of 11 strains LAB belonging to *Lactobacillus rhamnosus* (CIF1A1, CIF1A2, CIF1A9 and CIF1A10), *Lactobacillus casei* (NIF1A7 and NIF1AN12) and *Lactobacillus plantarum* (CIF17A2, CIF17A4, CIF17A5, CIF17AN2 and CIF17AN8) and 5 strains of bifidobacteria belonging to *Bifidobacterium longum* subsp. *longum* (NIF3AN3 and NIF7AN2) and *Bifidobacterium bifidum* (NIF7AN3, NIF7AN5 and NIF7AN5) isolated from infant feces was assessed by using broth microdilution assay. Sensitivity of probiotic bacteria to different antibiotics was expressed in term of minimum inhibitory (MIC) and bactericidal (MBC) concentrations.

Susceptibility of LAB and bifidobacteria isolated from infant feces to several antibiotics inhibiting cell wall synthesis (ampicillin, penicillins and vancomycin) are summarized in Table 12. All tested strains of LAB and bifidobacteria were susceptible to ampicillin and penicillin G. In addition, all strains of bifidobacteria were susceptible to vancomycin. On the contrary, all LAB strains including *L. rhamnosus* strain CIF1A1, CIF1A2, CIF1A9 and CIF1A10, *L. casei* strain NIF1A7 and NIF1AN12, *L. plantarum* strain CIF17A2, CIF17A4, CIF17A5, CIF17AN2, and CIF17AN8 reported in this study were highly resistant to vancomycin at the concentration up to 256 μg ml^{-1}.

Table 13 summarized the susceptibility of LAB and bifidobacteria to several antibiotics inhibiting protein synthesis (erythromycin, streptomycin, chloramphenicol, and tetracycline). None of the probiotic strains showed resistance to erythromycin and tetracycline. *Lactobacillus rhamnosus* (CIF1A1, CIF1A9 and CIF1A10) were resistant to streptomycin and *L. rhamnosus* CIF1A9 and *L. casei* NIF1A7 were resistant to chloramphenicol while, other strains of LAB and all strains of bifidobacteria were susceptible to these antimicrobial agents.

All strains of LAB and 4 out of 5 strains of bifidobacteria (*B. longum* subsp. *longum* NIF7AN2 and *B. bifidum* NIF7AN3, NIF7AN5 and NIF7AN10) were resistant to polymyxin B, a compound that is almost exclusively active against Gram-negatives with MIC values >265 μg ml^{-1} and ≥128 μg ml^{-1}, respectively (Table 14), indicating that this antibiotic is not active for these bacteria. In contrast, all strains of LAB and bifidobacteria were susceptible to rifampicin, a compound which inhibits DNA transcription by binding to

the DNA-dependent RNA polymerase with MIC value ranging from 0.125-2 µg ml^{-1} (Table 15).

Table 11. Microbiological breakpoints categorizing bacteria as resistant (mg l^{-1}). Strains with MIC higher than the breakpoint are considered as resistant.

Lactic acid bacteria	Ampicillin	Vancomycin	Gentamicin**	Kanamycin**	Erythromycin	Streptomycin**	Clindamycin	Quinupristin	Tetracycline	Chloramphenicol
Lactobacillus obligate homofermentative	1	2	16	16	16	1	1	4	4	4
Lactobacillus helveticus	1	2	16	16	16	1	1	4	4	4
Lactobacillus acidophilus group	1	2	16	16	16	1	1	4	4	4
Lactobacillus delbrueckii	1	2	16	16	16	1	1	4	4	4
Lactobacillus obligate heterofermentative	2	n.r.	16	16	64	1	1	4	8	4
Lactobacillus reuteri	2	n.r.	8	16	64	1	1	4	16	4
Lactobacillus fermentum	1	n.r.	16	32	64	1	1	4	8	4
Lactobacillus facultative heterofermentative*	4	n.r.	16	64	64	1	1	4	8	4
Lactobacillus plantarum	2	n.r.	16	64	n.r.	1	1	4	32	8
Lactobacillus rhamnosus	4	n.r.	16	64	32	1	1	4	8	4
Lactobacillus paracasei	2	n.r.	32	64	n.r.	1	1	4	4	4
Bifidobacterium	2	2	64	n.r.	128	0.5	0.25	1	8	4
Enterococcus	4	4	32	512	128	4	4	4	2	8
Pediococcus	4	n.r.	16	64	64	1	1	4	8	4
Leuconostoc	2	n.r.	16	16	64	1	1	4	8	4
Lactococcus lactis	2	4	32	64	64	2	4	4	4	8
Streptococcus thermophilus	2	4	32	64	64	2	2	4	4	4
Bacillus spp.	n.r.	4	4	8	8	4	4	4	8	8
Propionibacterium	2	4	64	64	64	0.5	0.25	0.5	2	2
Other Gram (-)	1	2	4	16	8	0.5	0.25	0.5	2	2

Source : Radulović *et al*. (2012)

n.r. not required

*including *Lactobacillus salivarius*

**possible interference of the growth medium

Table 12. Susceptibility of lactic acid bacteria and bifidobacteria isolated from infant feces to several antibiotics inhibiting cell wall synthesis (β-lactams: ampicillin, penicillins and vancomycin) by using broth microdilution assay.

Tested strains	Antimicrobial agent (μg ml^{-1})					
	Ampicillin		Penicillin		Vancomycin	
	MIC	MBC	MIC	MBC	MIC	MBC
L. rhamnosus CIF1A1	1s	4	0.5s[1]	1	>256r	>256
L. rhamnosus CIF1A2	1s	2	0.25s[1]	0.5	>256r	>256
L. rhamnosus CIF1A9	1s	2	0.25s[1]	0.5	>256r	>256
L. rhamnosus CIF1A10	1s	2	0.25s[1]	0.5	>256r	>256
L. casei NIF1A7	2s	8	0.5s[1]	0.5	>256r	>256
L. casei NIF1AN12	2s	4	0.5s[1]	2	>256r	>256
L. plantarum CIF17A2	1s	1	2s[1]	4	>256r	>256
L. plantarum CIF17A4	0.5s	1	2s[1]	4	>256r	>256
L. plantarum CIF17A5	0.5s	0.5	2s[1]	4	>256r	>256
L. plantarum CIF17AN2	0.5s	1	1s[1]	4	>256r	>256
L. plantarum CIF17AN8	0.5s	1	1s[1]	4	>256r	>256
B. longum subsp. *longum* NIF3AN3	0.125s	0.25	8s[2]	16	0.5s	1
B. longum subsp. *longum* NIF7AN2	0.125s	0.25	16s[2]	16	0.5s	2
B. bifidum NIF7AN3	0.125s	0.25	4s[2]	8	0.25s	1
B. bifidum NIF7AN5	0.25s	0.5	4s[2]	8	0.25s	0.5
B. bifidum NIF7AN10	0.25s	0.5	1s[2]	2	0.25s	1

[1] Breakpoint derived from Klare *et al*. (2007)

[2] Breakpoint derived from Moubareck *et al*. (2005)

s = sensitive

r = resistance

Table 13. Susceptibility of lactic acid bacteria and bifidobacteria isolated from infant feces to several antibiotics inhibiting protein synthesis (erythromycin, streptomycin, chloramphenicol and tetracycline) by using broth microdilution assay.

Tested strains	Antimicrobial agent (μg ml^{-1})							
	Erythromycin		Stretomycin		Chloramphenicol		Tetracycline	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
L. rhamnosus CIF1A1	0.5s	2	128r	128	4s	16	2s	4
L. rhamnosus CIF1A2	0.125s	0.5	32s	128	4s	16	1s	4
L. rhamnosus CIF1A9	0.25s	0.5	64r	128	8r	16	2s	4
L. rhamnosus CIF1A10	0.25s	0.5	64r	128	4s	16	2s	4
L. casei NIF1A7	0.25s	1	128s*	128	8r	32	2s	8
L. casei NIF1AN12	0.5s	0.5	128s*	256	4s	16	2s	4
L. plantarum CIF17A2	0.5s	2	256s*	256	8s	32	16s	64
L. plantarum CIF17A4	0.25s	1	128s*	256	4s	16	16s	64
L. plantarum CIF17A5	0.25s	1	128s*	256	4s	16	16s	64
L. plantarum CIF17AN2	0.25s	1	128s*	128	4s	16	8s	64
L. plantarum CIF17AN8	0.25s	1	128s*	256	4s	8	16s	64
B. longum subsp. *longum* NIF3AN3	0.125s	0.25	0.125s	0.125	0.125s	0.125	0.125s	0.125
B. longum subsp. *longum* NIF7AN2	0.125s	0.25	32s	32	0.125s	0.125	0.125s	0.125
B. bifidum NIF7AN3	0.125s	0.25	128s	256	1s	1	0.25s	0.5
B. bifidum NIF7AN5	0.125s	0.25	64s	128	0.5s	1	0.125s	0.25
B. bifidum NIF7AN10	0.25s	0.5	64s	128	0.5s	1	0.25s	0.25

*Breakpoint defined by Danielsen and Wind (2003).

s = sensitive

r = resistance

Table 14. Susceptibility of lactic acid bacteria and bifidobacteria isolated from infant feces to antibiotics inhibiting cytoplasmic function (polymyxin B) by using broth microdilution assay.

Tested strains	Antimicrobial agent (μg ml^{-1})	
	Polymyxin B	
	MIC	MBC
L. rhamnosus CIF1A1	>256	>256
L. rhamnosus CIF1A2	>256	>256
L. rhamnosus CIF1A9	>256	>256
L. rhamnosus CIF1A10	>256	>256
L. casei NIF1A7	>256	>256
L. casei NIF1AN12	>256	>256
L. plantarum CIF17A2	>256	>256
L. plantarum CIF17A4	>256	>256
L. plantarum CIF17A5	>256	>256
L. plantarum CIF17AN2	256	256
L. plantarum CIF17AN8	256	256
B. longum subsp. *longum* NIF3AN3	0.125	0.125
B. longum subsp. *longum* NIF7AN2	128	128
B. bifidum NIF7AN3	128	256
B. bifidum NIF7AN5	256	>256
B. bifidum NIF7AN10	256	>256

s = sensitive

r = resistance

Table 15. Susceptibility of lactic acid bacteria and bifidobacteria isolated from infant feces to antibiotics inhibiting nucleic synthesis (rifampicin) by using broth microdilution assay.

Tested strains	Antimicrobial agent (μg ml^{-1})	
	Rifampicin *	
	MIC	MBC
L. rhamnosus CIF1A1	0.125s	1
L. rhamnosus CIF1A2	0.125s	1
L. rhamnosus CIF1A9	0.125s	1
L. rhamnosus CIF1A10	0.125s	1
L. casei NIF1A7	0.125s	0.5
L. casei NIF1AN12	0.125s	0.5
L. plantarum CIF17A2	1s	2
L. plantarum CIF17A4	2s	4
L. plantarum CIF17A5	1s	2
L. plantarum CIF17AN2	0.5s	2
L. plantarum CIF17AN8	0.5s	1
B. longum subsp. *longum* NIF3AN3	0.5s	1
B. longum subsp. *longum* NIF7AN2	0.5s	1
B. bifidum NIF7AN3	0.125s	0.5
B. bifidum NIF7AN5	0.125s	0.5
B. bifidum NIF7AN10	0.125s	1

*Breakpoint defined by Danielsen and Wind (2003)

s = sensitive

r = resistance

3.5 Discussion

There is the growing concern about antibiotic resistance in probiotic bacteria. Especially, if encoded by genes located on mobile elements, may be potentially transferable from probiotic strains to commensal flora of human opportunistic bacteria. For this reason, the presence of acquired antibiotic resistance is one of the first criteria to be checked during the selection process of a potentially probiotic strain (Masco *et al*., 2006). However, antibiotic resistance may be intrinsic for bacterial species or a genus, and it is characterized by the ability of an organism to survive in the presence of certain

antimicrobial agents, due to its inherent characteristics of resistance. Intrinsic or "natural" resistance mechanisms involve the absence of the target antimicrobial agent, cell wall have poor premeability, production of enzyme to destroy the antimicrobial agent or presence of efflux mechanism. Intrinsic resistance and resistance by mutation are unlikely to be disseminated, although any gene responsible for intrinsic resistance may occur by insertion sequences (Radulović *et al*., 2012). Therefore, distribution between natural and acquired antibiotic resistance among the population of LAB is importance. Analysis of MIC and its distribution in defined species/antibiotic combinations helps to differentiate between these two resistant mechanisms. Theoretically, a uniform MIC distribution in the lower antibiotic concentration range indicates that all strains are susceptible, a uniform distribution with high MIC for all strains may be due to intrinsic trait; and biomodal MIC distribution indicates that the strains with high atypical MIC may have acquired resistance (Egervarn, 2009).

All tested strains of LAB and bifidobacteria were susceptible to ampicillin and penicillin G, which were cell wall synthesis inhibitor. This is similar result for other strains of LAB and bifidobacteria isolated from human gastrointestinal tract and dairy products (Majhenic and Matijasic, 2001; Delgado *et al.*, 2005; Zhou *et al*., 2005; D'Aimmo *et al.,* 2007). In addition, all strains of bifidobacteria were succeptible to vancomycin. In contrast, LAB (*L. rhamnosus*, *L. casei* and *L. plantarum*) were strongly resistant to vancomycin one of cell wall synthesis inhibition with MIC value >256 μg ml^{-1} in all strains. It is possible that our human isolates LAB, in particular lactobacilli, may have natural mechanisms reducing susceptibility to vancomycin. As well, *L. casei*, *L. rhamnosus*, *L. curvatus*, *L. plantarum* and *L. rhamnosus* GG that are commonly used in the food industry or naturally found in food raw material were intrinsically resistant to vancomycin (Tynkkynen *et al*., 1998). Vancomycin resistance of lactobacilli is intrinsic, due to the presence of D-Ala-D-lactate in their peptidoglycan instead of the normal dipeptide D-Ala-D-Ala (Handwerger *et al.*, 1994: Delgado *et al.*, 2005; Ammor *et al.*, 2008). Natural resistance to vancomycin in LAB was one property which is useful to separate *Lactobacillus*, *Pediococcus* and *Leuconostoc* spp. from other lactobacilli and Gram-positive bacteria (Hamiltor-Miller and Shah, 1998; Simpson *et al*., 1988). This type of resistance does not seem to pose a problem since it is different from the inducible, transferable mechanism observed in other bacteria (Delgado *et al.,* 2005).

All strains of human isolates LAB and bifidobacteria were susceptible to erythromycin and tetracycline. Similarly, all strains of tested bifidobacteria were susceptible to chloramphenicol and streptomycin which interacts directly by impairing bacterial protein synthesis through binding to small ribosomal subunit (Mingeot-Leclercq *et al*., 1999). On the other hand, 3 out of 4 strains of *L. rhamnosus* (CIF1A1, CIF1A9 and CIF1A10) were strongly resistant to aminoglycoside streptomycin. The minimum inhibitory concentration value of these resistant strains varied from 64-128 μg ml^{-1}. According to the knowledge of the distribution of MIC, it was possible that the aminoglycoside streptomycin resistance in 3 strains of *L. rhamnos*us was acquired resistant. There are three mechanisms of aminoglycoside resistance; a decreased uptake and/or accumulation of the drug in bacteria by membrane impermeabilization, alteration of the ribosomal binding site and bacterial expression of enzymes which modify antibiotic structure and thereby inactivate it (Mingeot-Leclercq *et al*., 1999). The first two mechanisms seem to be safe due to they located on chromosome, whereas the genes encoding for aminoglycoside modifying enzymes are usually found on plasmids and transposons (Mingeot-Leclercq *et al*., 1999). The pattern of MIC distribution of streptomycin in our *L. rhamnosus* was correlated to the MIC range of tetracycline, erythromycin, clindamycin and streptomycin in some tested LAB and bifidobacteria isolated from different origin and geographical location of which showed a bimodal distribution, suggesting that these bacteria possess acquired antibiotic resistance. However, after screening for resistance genes, it was found that they located on the bacterial chromosome, except for tet(M), which was identified on plasmids in *Lactococcus lactis* (Ammor *et al*., 2008). As well, streptomycin resistance in commercial LAB present in microbial foods and drug additive has been reported however the streptomycin resistance gene was not detected (Liu *et al*., 2009). Therefore, it was possible that streptomycin resistance in our *L. rhamnosus* seem to be intrinsic. Intrinsic resistance to aminoglycoside antibiotics has been observed by many other investigators (Bayer *et al*., 1980; Charteris *et al*., 1998; Katta *et al*., 2001; Danelson and Wind, 2003; Elkins and Mullis, 2004; Zhou *et al*., 2005). Intrinsic resistance to aminoglycosides in lactobacilli could be confirmed by losing of resistance when they presented in conjugated bile salts suggesting that membrane impermeability plays an important role in this intrinsic resistance (Bayer *et al*., 1980; Charteris *et al*., 2000; Elkins and Mullis, 2004). Moreover, our results also showed that all

strains of human isolates both LAB and bifidobacteria were sensitive to streptomycin aminoglycoside when the breakpoint defined by Danelson and Wind (2003) was used.

In addition, our human isolates *L. rhamnosus* CIF1A9 and *L. casei* NIF1A7 exhibited intermediate resistant to chloramphenicol with MIC value 8 μg ml^{-1} which was just a bit above breakpoint (4 μg ml^{-1}). However, the proposed breakpoint from the published body of research and from nation and European moitoring procedures (4 μg ml^{-1}) is very low when compared to Danielson and Wind (2003) (16 μg ml^{-1}). Therefore, following this break point, our human isolates *L. rhamnosus* CIF1A9 and *L. casei* NIF1A7 were susceptible to chloramphenicol.

Polymyxin B is a cell membrane inhibitor, its roles are known to disorganize the structure or inhibit the function of bacterial membrane (Domig *et al*., 2007). Unfortunately, our LAB and bifidobacteria were strongly resistant to this antibiotic. Polymyxin B binds to membrane phospholipids and thereby interferes with membrane function. The resistance to this antibiotic of bifidobacteria was reported by Masco *et al*. (2006). In addition, polymyxin B resistance also found in many commercial probiotic including *L. rhamnosus* HN001 (DR20^{TM}), *Lactobacillus acidophilus* HN017 and *Bifidobacterium lactis* HN019 (DR10^{TM}) (Zhou *et al*., 2005). Polymyxin resistance in Gram-positive bacteria seems to be intrinsic but it poses potent bactericidal activity against most Gram-negative bacteria (Viljanen and Baara, 1984).

Our human isolates LAB and bifidobacteria were susceptible to rifampicin which is nucleic acid inhibitor. It changes the RNA polymerase encoded by chromosomal mutations that occur rapidly in the presence of the drug. This was in agreement with *Lactobacillus* and *Bifidobacterium* isolated from commercial dairy, pharmaceutical product, animal and probiotic products (Masco *et al*., 2006; D'Aimmo *et al*., 2007).

3.6 Conclusion

None of our human isolates lactobacilli and bifidobacteria was resistant to ampicillin, penicillin G, erythromycin, tetracycline and rifampicin. All strains of LAB seemed to intrinsic resistance to vancomycin and polymyxin B. *Lactobacillus rhamnosus* CIF1A1, CIF1A9 and CIF1A10 were strongly resistant to streptomycin. Therefore, for safety concerns, the experiment for determination plasmid encoded antibiotic resistance gene in the latter group should be performed.

CHAPTER 4

ADHESION ABILITY OF LACTOBACILLI AND BIFIDOBACTERIA ISOLATED FROM INFANT FECES AND ASSESSMENT OF CELL SURFACE PROPERTIES

4.1 Abstract

Adhesion ability of probiotic to the intestinal mucosa is considered to be the prerequisite for colonization of probiotic and can protect against gastrointestinal pathogens infection. Our lactobacilli and bifidobacteria were investigated for their adhesion ability to mucin before and after exposing to simulated upper human gastrointestinal tract conditions and their ability to inhibit pathogen adhesion to mucin was also investigated. Afterward, cell surface characteristic of mucin adhesive strains was partially characterized. Seven strains of lactobacilli and 5 strains of bifidobacteria were able to bind to porcine mucin at different levels. *Lactobacillus casei* and *Bifidobacterium bifidum* showed distinctly adhesion capacity higher than well-known mucin adhesive *Lactobacillus plantarum* 299V ($P<0.05$) whereas, 5 strains of *Lactobacillus plantarum* and 2 strains of *Bifidobacterium longum* subsp. *longum* displayed moderate adhesion. These mucin adhesive strains still maintained the adhesion ability after passing through simulated gastric fluid and small intestinal fluid. Moreover, the mucin adhesive lactobacilli and bifidobacteria were able to inhibit adhesion of enteropathogens to mucin by competitive adhesion. After partial characterization, it was found that cell surface protein extremely involved in adhesion of high mucin adhesive *B. bifidum*. In contrast, no protein component involved in adhesion of *L. casei* one of high mucin adhesive bacteria. However, both high mucin adhesive bacteria showed the same cell physicochemical characteristic with strong hydrophobicity and electron donor properties. While, some of bacterial surface proteins involved in adhesion of intermediate mucin adhesive *L. plantarum*. The physicochemical characteristics of this intermediate mucin adhesive bacterium were moderate cell surface hydrophobicity and electron acceptor properties.

4.2 Introduction

Probiotics are live microbial food supplement that benefit the host health by restoring balanced microflora environment (Floch and Hong-Curtiss, 2001; Holzapfel and Schillinger, 2002). They have shown to inhibit the growth of pathogenic bacteria by secretion of inhibitory substances (organic acids, bacteriocins) and by competitive adhesion to the epithelium (Urdaci *et al*., 2007). Adhesion of probiotic to human mucosa and epithelium cell has been suggested as the first prerequisite for probiotic action. In addition, it is also considered important to immune modulation, enhancing the healing of damaged gastric mucosa, antagonism against enteric pathogens and also crucial for transient colonization (Collado *et al*., 2006; Collado *et al*., 2007a; Collado *et al*., 2007b; Ouwehand *et al*., 2001).

The mucin is a hydrated polymeric gel with a thickness of 50-800 µm, which is composed of two layers: a loosely adherent layer removable by suction and a layer firmly attached to the mucosa. It is normal cover the mucosal surface. The mucin is secreted by goblet to cover the cells and is composed of proteins, carbohydrates and lipids. Its main constituent is a glycoprotein (mucin) (Swidsinski *et al*., 2007). It acts as a barrier to protect the host from harmful antigens and also provides a habitat and nutrients for the intestinal microflora (Urdaci *et al*., 2007). This layer is the first physical barrier to host-cell stimulation by bacteria in the gut. Adhesion to this mucin is therefore the first step required for probiotic organisms to interact with host cells and elicit any particular responses.

The mechanisms of adhesion are poorly understood. Several mechanisms mediate the adherence of bacteria to host tissues (Strus *et al*., 2001). Bacteria adhere initially to GI surface by non-specific physical interaction. The physicochemical surface characteristic of bacterial cell and solid surface influence the attractive and repulsive forces between these surfaces and therefore play a crucial role in adhesion (Hamadi *et al*., 2012). These characteristics include surface charge, hydrophobicity and electron donor-acceptor properties (acid-base). High cell surface hydrophobicity is attractive and may favor the colonization of mucosal surfaces. Therefore hydrophobic characteristic could primarily play a role in the adhesion of bacteria to epithelial cells and extracellular matrix proteins (Pérez *et al*., 1998; Kos *et al*., 2003; Schillinger *et al*., 2005). Hydrophobicity and electron donor-acceptor properties favor bacterial adhesion has been reported (Loosdrecht *et al*.,

1987; Bellon-Fontaine *et al.*, 1996; Owehand *et al.*, 1999; Briandet *et al.*, 2001; Lee and Puong, 2002; Sinde and Carballo, 2007; Muňoz-Provencio *et al*., 2009). In addition, adhesion of bacteria to intestinal mucin also involved in particular molecules. It has been reported that proteins such as S-layer proteins as well as other cell-surface associated proteins can be involved in adhesion (Horie *et al*., 2002; Wang *et al*., 2010b).

Therefore, to obtain proper probiotic bacteria, we examined the adhesion ability of probiotic lactobacilli and bifidobacteria isolated from infant feces to mucin, their competitive adhesion against enteropathogens, and their adhesion ability after exposure to simulated human stomach fluid and small intestinal fluid. The partial characterization of mucin adhesive probiotic bacteria was also determined.

4.3 Materials and methods

4.3.1 Bacterial strains

The 11 strains of lactobacilli and 5 strains of bifidobacteria were isolated from feces obtained from 2-5 months and 2-6 days old, healthy breast fed, natural born and caesarean born infants. The control *Lactobacillus plantarum* 299V was obtained from Assoc. Prof. Dr. Sunee Nitisinprasert, Kasetsart University, Bangkok, Thailand. Lactobacilli were cultivated routinely in MRS broth (Himedia, Mumbai, India). Bifidobacteria were cultured in MRS broth containing 0.05% L-cysteine (Sigma, Japan) and 1 mg l^{-1} resazurin (Sigma, US) contained in injection vial, which was sealed with rubber stopper and secured by aluminum cap, after being filled with nitrogen gas to achieve anaerobic condition. The tested lactobacilli and bifidobacteria were grown in MRS broth to the late log phase (15 h for *Lactobacillus plantarum*; 18 h for *Lactobacillus casei*; 24 h for *Bifidobacterium bifidum*; 32 h for *Bifidobacterium longum* subsp. *longum*). *Escherichia coli* TISTR 780 and *Staphylococcus aureus* TISTR 1466 were obtained from Microbiological Resources Centre (MIRCEN), Thailand. *Escherichia coli* O157: H7 DMST 12743 was from the Department of Medical Science, Ministry of Public Health, Thailand. *Listeria monocytogenes, Shigella sonei, Shigella flexneri, Salmonella enterica* subsp. *enterica* serovar Typhimurium SA2093 and *Salmonella* Paratyphi A were derived from the Microbiological Laboratory of Songklanakarind Hospital, Songkhla, Thailand. All

pathogens were grown in Muller Hinton broth (MHB; Himedia, Mumbai, India) at 37 °C for 8 h (late log phase).

4.3.2 Preparation of mucin coating plate

The adhesion test was based on the method of Tallon and colleagues (2007). Partially purified porcine gastric mucin type III (Sigma, Louis, USA) was dissolved in phosphate buffer saline (PBS, pH 7) at a concentration of 10 mg ml^{-1} One hundred microliter of the mucin solution was added to a sterilized polystyrene microtiter well plate (Maxisorp Nunc, Roskilde, Denmark). The plate was left at 37 °C for 1.5 h and then at 4 °C overnight to allow immobilization of the mucin. The wells were then washed twice with 200 µl of PBS followed by addition of 200 µl of 2% (w/v) bovine serum albumin (BSA) (Sigma) solution. The plate was then further incubated for 4 h at 4 °C and again washed twice with PBS. A minimum of four replicates were used to estimate the adhesion of the tested strain. A well-known mucin adhesive *Lactobacillus plantarum* 299V was used as a positive control (Tallon *et al.*, 2007).

4.3.3 Adhesion assay

The late log phase of bacterial culture (1 ml) was sampled and centrifuged at 10,000 rpm for 10 minutes and the pellet was washed twice with 1 ml of sterile PBS and resuspended in the same buffer and then adjusted to obtain concentration of 1×10^{7} CFU ml^{-1} (N_0). One hundred microliter of the bacterial suspension was added to mucin coated well. The plates were incubated for 1 h at 37 °C. Microtiter plate wells were washed 12 times with 200 µl of sterile PBS to remove unbound bacteria. Thereafter, 0.05% (v/v) of triton X-100 solution was added to each well (200 µl) in order to detach bound bacteria, and then microplates were incubated at room temperature for 2 h with gentle agitation. An aliquot from each well was removed and diluted to obtain appropriate dilution thereafter; a number of adhesive bacteria were counted using spread plate method for LAB and pour plate with MRS agar for bifidobacteria (N_1). Percentage of adhesion was calculated as equation below.

$$\text{Adhesion (\%)} = \frac{\log \text{CFU } N_1}{\log \text{CFU } N_0} \times 100$$

4.3.4 Adhesion ability of probiotic bacteria after exposure to upper gastrointestinal condition

The late log phase of lactobacilli and bifidobacteria was centrifuged and washed twice with PBS. The cell pellets were exposed to simulated gastric condition (0.85% NaCl containing 3 g l^{-1} pepsin (Sigma, Geramany) and adjusted to pH 2 with HCl) and simulated intestinal condition (0.3% ox-gall bile and 3 mg ml^{-1} pancreatin (Sigma, USA)). The test tubes were incubated at 37 °C for 3 h and 6 h with gentle agitation for acid and bile condition, respectively. After incubation, cell suspensions were centrifuged and adjusted to achieve concentration of about 1×10^7 CFU ml^{-1} (N_0) and used for adhesion assay as in 4.3.3. The number of adhesive bacteria was counted (N_1) and calculated the percentage of of adhesion according to the equation described in 4.3.3. Adhesion of LAB and bifidobacteria exposure to 0.85% NaCl for 3 and 6 h was used as control.

4.3.5 Inhibition of pathogen adhesion

The mucin adhesive lactobacilli and bifidobacteria were tested for their ability to inhibit the adhesion of pathogen to mucin. The six pathogenic bacteria, including *E. coli* TISTR 780, *S. aureus* TISTR 1466, *S. sonei*, *S. flexneri*, *Sal.* Typhimurium SA2093 and *Sal.* Paratyphi A were cultured in MHB to the late log phase. After incubation, the tested bacteria were centrifuged thereafter washed twice with PBS and then resuspended in the same buffer. The tested pathogenic bacteria were adjusted to achieve final concentration of 1×10^7 CFU ml^{-1}. Then equal volume of pathogen and probiotic bacteria (prepared in 4.3.2) was mixed together. The ability of lactobacilli and bifidobacteria to inhibit the adhesion of pathogen to mucin was evaluated by simultaneous addition of pathogen-probiotic mixture (100 µl) to the mucin coated well plates. After incubation at 37°C for 1 h, unbound bacteria were removed by washing twice with 200 µl of PBS. Then, mucin adhesive bacteria were detached by treating with Triton X-100. The adhesive pathogens were counted on MHA. Lactobacilli and bifidobacteria could not be able to grow on this medium. This was confirmed by control plate of MHA inoculated with lactobacilli and bifidobacteria. The percentage of competitive inhibition of probiotic against pathogen was calculated based on the difference in number of adhesive pathogen in the presence (N_1) and absence of lactobacilli or bifidobacteria (N_0).

4.3.6 Cell surface characteristic and mechanism of adhesion

4.3.6.1 Determination of cell surface protein involvement in adhesion of lactobacilli and bifidobacteria

The early stationary phase of lactobacilli and bifidobacteria was centrifuged and washed twice with PBS. The cell pellets were treated with 5 mg ml^{-1} of trypsin solution (Sigma, Switzerland), 100 µg ml^{-1} of proteinase K, (Sigma, USA) (Ouwehand *et al*., 2001), and 5 M LiCl (Frece *et al*., 2005). Both enzymatic pretreatments were incubated at 37°C for 1 h whereas LiCl pretreatment bacterial cell was incubated at 4°C for 30 minute with gentle agitation. Then, cell suspensions were centrifuged and washed twice with PBS followed by resuspended in PBS and adjusted to achieve final concentration of 1×10^7 CFU ml^{-1} (N_0) and use for adhesion assays (4.3.3). Number of adhesive bacteria was counted (N_1) and percentage of adhesion was calculated according to the equation as described in 4.3.3. Bacterial exposed to PBS was run as control.

4.3.6.2 Physicochemical characteristic of mucin adhesive strain

The bacterial adhesion to hydrocarbon (BATH) was used to determine bacterial cell surface hydrophobicity. The method was performed according to Muño-Provencio and team (2009) with some modifications. The early stationary phase of lactobacilli and bifidobacteria was centrifuged. Cell pellets were washed with PBS and adjusted to obtain final OD_{600} of ~0.5 (A_0). The bacterial suspensions (1.5 ml) were mixed with 0.5 ml of *n*-hexadecane, ethyl acetate or chloroform for 1 minute at full speed of vortex mixer. The mixtures were left to stand for 20 minute to allow the phase separation and the aqueous phase was removed to measure optical at 600 nm (A_1). The percentage of bacteria adhere to solvent was calculated from: $\%\ \text{Adhesion} = [(A_0-A_1)/A_0] \times 100$.

4.4 Results

4.4.1 Mucin adhesion of lactobacilli and bifidobacteria isolated from infant feces

Porcine mucin type III was used as model for testing the adhesion ability of lactobacilli and bifidobacteria isolated from infant feces. Bacterial strains which their adhesion ability was higher than mucin adhesive *L. plantarum* 299V were defined as high mucin adhesive strain. The result was shown in Figure 10. All tested strains were able to

adhere to mucin. High mucin adhesive bacteria were found in *L. casei* (NIF1A7 and NIF1AN12) and *B. bifidum* (NIF7AN3, NIF7AN5 and NIF7AN10). The adhesion capacity of these strains was 59.66, 59.23, 57.27, 72.51 and 66.58%, respectively which was significantly (*P<0.05*) higher than the adhesive strain of *L. plantarum* 299V (48.49%). Whereas, all strains of *L. plantarum* and 2 strains of *B. longum* subsp. *longum* were moderate mucin adhesive bacteria with adhesion levels ranged from 37.02% to 46.79% and 34.31% to 35.84%, respectively.

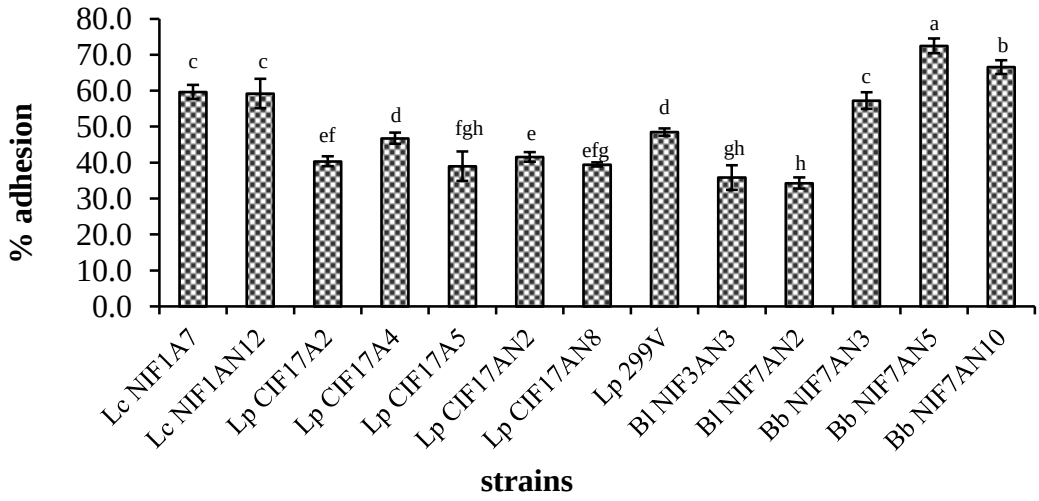


Figure 10. Adhesion to porcine gastric mucin of lactobacilli and bifidobacteria isolated from infant feces and mucin adhesive *L. plantarum* 299V (positive control).

4.4.2 Adhesion ability of lactobacilli and bifidobacteria after exposure to simulate upper gastrointestinal tract conditions

Before reaching the target site, probiotic bacteria have to exposure to extreme conditions in the upper part of human gastrointestinal tract which may be influent on their adhesion. Therefore, in this study we evaluated the effect of low pH condition in stomach and high bile salt condition in upper small intestine on the adhesion ability of lactobacilli and bifidobacteria isolated from infant feces. Influence of simulated human gastrointestinal tract condition on adhesion ability of tested lactobacilli and bifidobacteria was shown in Figure 11. The extreme conditions in stomach and small intestine had no negative effect on mucin adhesion of *L. plantarum* strain CIF17A4, CIF17A5, CIF17AN2, and CIF17AN8. In contrast, such extreme condition significantly (*P<0.05*) deteriorated

mucin adhesion of *L. casei* (NIF1A7 and NIF1AN12), 3 strains of *B. bifidum* (NIF7AN3, NIF7AN5 and NIF7AN10) and also *L. plantarum* 299V. The adhesion capacity of *L. plantarum* CIF17A2 and *B. longum* subsp. *longum* NIF7AN3 was significantly damaged only after exposure to simulated upper part of small intestine condition.

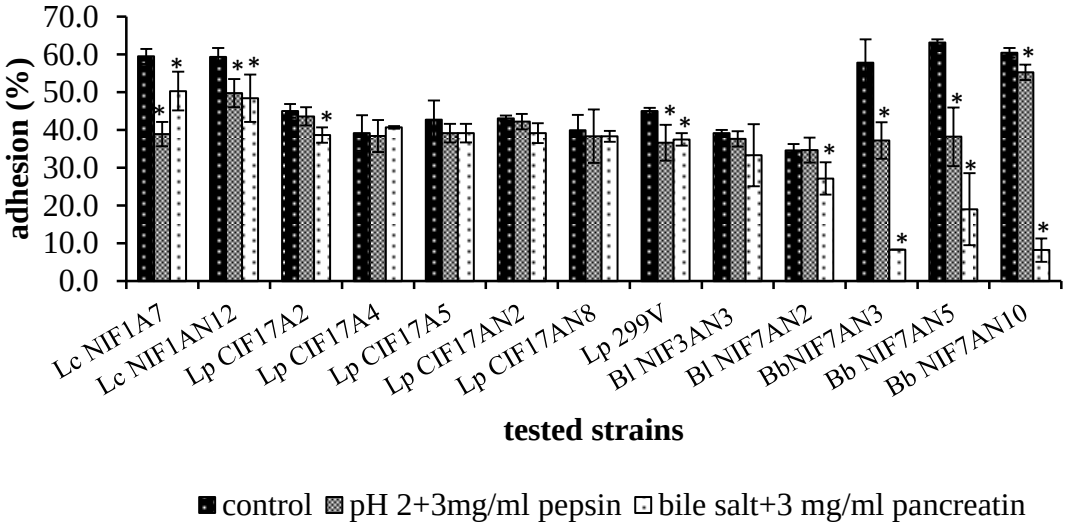


Figure 11. Adhesion of lactobacilli and bifidobacteria isolated from infant feces and mucin adhesive *L. plantarum* 299V (positive control) after exposing to low pH condition for 3 h or bile salt condition for 6 h. (*significant difference from control).

4.4.3 Inhibition of pathogen adhesion to mucin

To study competitive adhesion to mucin between 12 mucin adhesive probiotic bacteria and various enteropathogens, the equal concentrations of both bacteria were simultaneously added to mucin coated well plate. The level of competitive inhibition was shown in Table 16. The degree of adhesive inhibition varied dependent on strain. All strains of bifidobacteria were able to inhibit the adhesion of *E. coli* TISTR 789 (31.71-43.81%), *S. sonei* (33.67-50.55%), *S. flexneri* (32.49-38.46%), *Sal.* Paratyphi A (13.35-38.69%), *Sal.* Typhimurium SA2093 (1.16-30.51%), *S. aureus* (14.37-37.96%) ($P<0.05$) which was significantly higher than *L. plantarum* 299V (-1.16-7.54%). All strains of mucin adhesive lactobacilli also exhibited adhesive inhibition toward almost tested pathogenic bacteria except *E. coli* TISTR 780 and *S. sonnei* with inhibition level varied from 0.37-6.52%.

4.4.4 Influence of protein and S-layer protein on mucin adhesion of lactobacilli and bifidobacteria

To investigate protein involvement in the adhesion ability of 12 mucin adhesive lactobacilli and bifidobacteria, the tested bacteria were treated with typsin, proteinase K and 5 M LiCl and then determination for their adhesion to mucin. The adhesion capacity of strong mucin adhesive *B. bifidum* strain NIF7AN3, NIF7AN5, NIF7AN10 and *L. casei* NIF1AN12 was dramatically reduced after pretreatment with proteolytic enzyme (Figure 12). However, only *B. bifidum* NIF7AN3 was significantly decreased its adhesion to mucin after exposure to both trypsin and proteinase K. Only 3 strains (*L. plantarum* CIF17A5, *L. plantarum* CIF17AN2, and *B. longum* subsp. *longum* NIF7AN2) out of 7 strains of intermediate mucin adhesive probiotic bacteria and also *L. plantarum* 299V were significantly lowered their adhesion ability after incubation with either proteinase K or trypsin. Whereas, both enzymatic pretreatment had no negative effect on adhesion of *L. casei* NIF1A7 (high mucin adhesive stain), *L. plantarum* strain CIF17A2, CIF17A3, CIF17AN8, and *B. longum* subsp. *longum* NIF3AN3 (intermediate mucin adhesive strain). The adhesion ability of all strain of both high and intermediate mucin *adhesive B. bifidum*, *B. longum* subsp. *longum* and *L. plantarum* significantly decreased after LiCl exposure which is S-layer protein extraction agent (Figure 13). Especially, high mucin adhesive *B. bifidum*, over 50% reductions in adhesion was observed indicating that S-layer protein maybe extremely involve in adhesion in this bacteria. In contrast, LiCl pretreatment had no negative effect on high mucin adhesive *L. casei*.

Table 16. Competitive exclusion of probiotic strains against foodborne pathogens in adhesion to porcine intestinal mucin.

strains	% competitive exclusion*					
	E. coli TISTR 780	*S. sonei*	*S. flexneri*	*Sal.* Paratyphi A	*Sal.* Typhimurium SA2093	*S. aureus* TISTR 1466
L.casei NIF1A7	1.92 ± 2.59^{d}	-1.49 ± 3.14^{de}	3.91 ± 1.40^{c}	13.47 ± 3.08^{c}	12.74 ± 5.15^{cd}	10.93 ± 2.90^{cd}
L. casei NIF1AN12	1.26 ± 4.93^{d}	2.09 ± 3.33^{cd}	0.37 ± 0.00^{c}	4.09 ± 2.63^{d}	8.20 ± 3.07^{cde}	5.85 ± 2.52^{d}
L. plantarum CIF17A2	-7.98 ± 0.97^{e}	6.75 ± 0.00^{c}	2.81 ± 2.53^{c}	3.17 ± 1.45^{d}	5.89 ± 2.44^{de}	4.92 ± 1.24^{d}
L. plantarum CIF17A4	-4.01 ± 3.19^{de}	3.43 ± 2.87^{c}	0.37 ± 0.00^{c}	6.66 ± 0.15^{d}	9.89 ± 3.99^{cd}	5.70 ± 0.62^{d}
L. plantarum CIF17A5	-3.33 ± 3.66^{de}	3.43 ± 2.87^{c}	2.05 ± 2.91^{c}	5.00 ± 3.03^{d}	7.96 ± 1.47^{cde}	5.29 ± 0.68^{d}
L. plantarum CIF17AN2	-10.20 ± 3.22^{f}	-3.58 ± 1.58^{e}	5.41 ± 0.00^{c}	6.93 ± 3.16^{d}	16.52 ± 1.99^{bc}	5.78 ± 1.88^{d}
L. plantarum CIF17AN8	-10.45 ± 0.95^{f}	2.26 ± 4.27^{cd}	2.81 ± 2.53^{c}	6.66 ± 0.15^{d}	16.16 ± 2.45^{bc}	5.30 ± 0.93^{d}
L. plantarum 299V	1.88 ± 6.42^{d}	2.37 ± 2.58^{cd}	-1.16 ± 1.85^{c}	6.92 ± 3.27^{d}	7.54 ± 1.65^{de}	6.09 ± 2.79^{d}
B. longum subsp. *longum* NIF3AN3	43.81 ± 4.42^{a}	35.33 ± 1.24^{b}	34.31 ± 2.40^{a}	18.49 ± 0.12^{b}	1.16 ± 0.32^{e}	14.37 ± 2.82^{c}
B. longum subsp. *longum* NIF7AN2	40.74 ± 1.87^{ab}	34.38 ± 1.99^{b}	34.80 ± 2.94^{a}	17.78 ± 2.01^{b}	30.51 ± 8.41^{a}	37.96 ± 1.78^{a}
B. bifidum NIF7AN3	36.69 ± 0.64^{bc}	35.39 ± 1.87^{b}	38.46 ± 0.60^{a}	13.35 ± 0.55^{c}	22.86 ± 6.62^{ab}	23.59 ± 0.49^{b}
B. bifidum NIF7AN5	31.71 ± 1.44^{c}	33.67 ± 0.37^{b}	32.49 ± 2.04^{ab}	35.33 ± 2.96^{a}	2.14 ± 3.44^{e}	22.51 ± 0.00^{b}
B. bifidum NIF7AN10	41.60 ± 3.44^{ab}	50.55 ± 2.50^{a}	34.06 ± 0.38^{b}	38.69 ± 2.14^{a}	29.86 ± 10.30^{a}	27.79 ± 1.25^{a}

* Competitive inhibition of the pathogens by LAB isolates. A high value represents a low number of adhered pathogens in the presence of LAB when compared to the adhesion of the pathogen alone. A low value represents a high number of adhered pathogens in the presence of LAB when compared to adhesion of the pathogen alone.

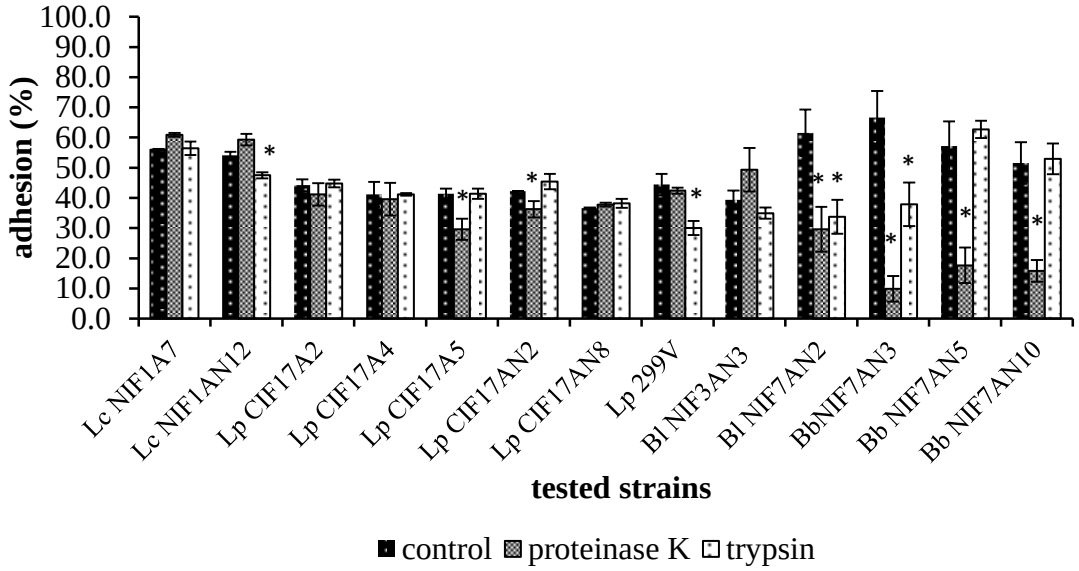


Figure 12. Effect of protease treatment on adhesion ability of lactobacilli and bifidobacteria isolated from infant feces and mucin adhesive *L. plantarum* 299V. (* significant difference from control).

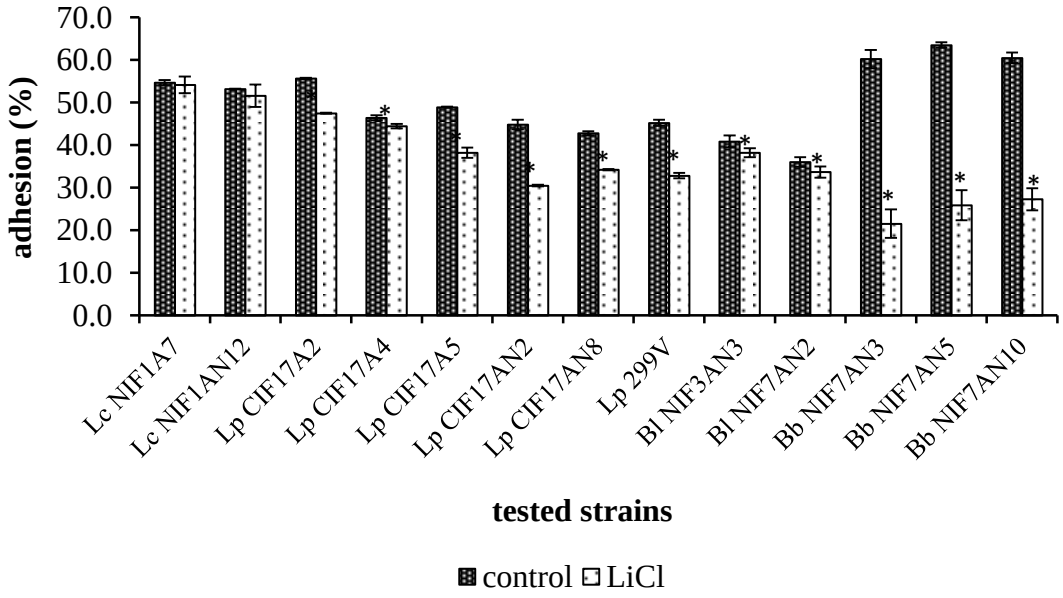


Figure 13. Effect of 5 M LiCl treatment on adhesion ability of lactobacilli and bifidobacteria isolated from infant feces and mucin adhesive *L. plantarum* 299V. (* significant difference from control).

4.4.5 Determination of physicochemical cell surface characteristic of mucin adhesive lactobacilli and bifidobacteria

It is important to know cell surface characteristic of mucin adhesive probiotic bacteria. Therefore, cell surface hydrophobicity and electron donor/electron acceptor properties were studied. We defined bacteria as high and intermediate cell surface hydrophobicity or electron donor-acceptor properties when the adhesion to *n*-hexadecane or chloroform and ethyl acetate was higher than 50% and 20%, respectively. Almost tested lactobacilli and bifidobacteria showed percentage of adherence to a polar solvent (7.20-55.25%) higher than well known mucin adhesive *L. plantarum* 299V (4.08%). High cell surface hydrophobicity was found in 3 strains of high mucin adhesive *B. bifidum* with affinity to *n*-hexadecane more than 50% (Figure 14). Intermediate cell surface hydrophobicity was found in 2 strains of *L. casei* and 5 strains of *L. plantarum* with 22.56-46.38% adhesion to *n*-hexadecane. Whereas, cell surface characteristic of *B. longum* subsp. *longum* and *L. plantarum* 299V (positive control) exhibited very low hydrophobicity with less than 10% specific to non-polar substance.

High mucin adhesive *L. casei*, *B. bifidum* and intermediate mucin adhesive *L. plantarum* 299V had more affinity to chloroform than ethyl acetate especially *L. casei* (NIF1A7 and NIF1AN12) and *B. bifidum* NIF7AN (Figure 15). Intermediate mucin adhesive *L. plantarum* showed more affinity to ethyl acetate. However, only *L. plantarum* CIF17A2 showed significantly affinity to ethyl acetate. Whereas, cell surface characteristic of intermediate mucin adhesive *B. longum* subsp. *longum* exhibited dual behavior by showing strong affinity to both chloroform and ethyl acetate.

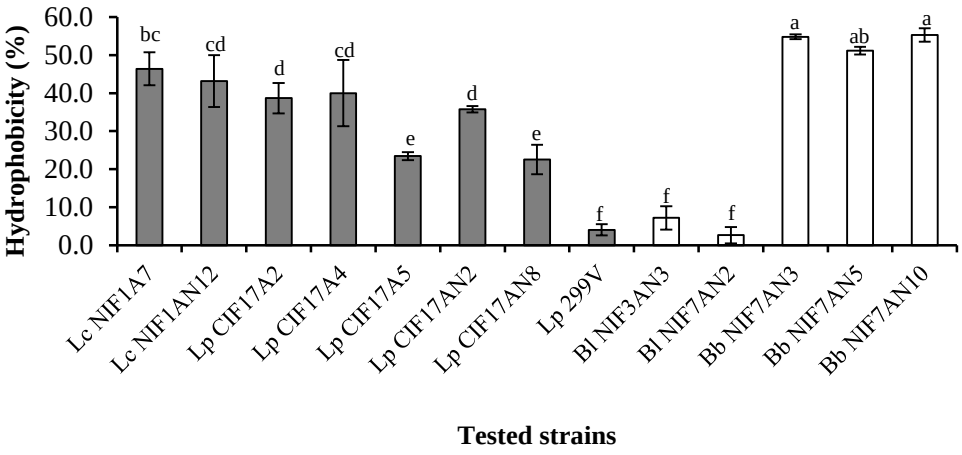


Figure 14. The percentages of adhesion to hexadecane of mucin adhesive lactobacilli and bifidobacteria isolated from infant feces and *L. plantarum* 299V (positive control).

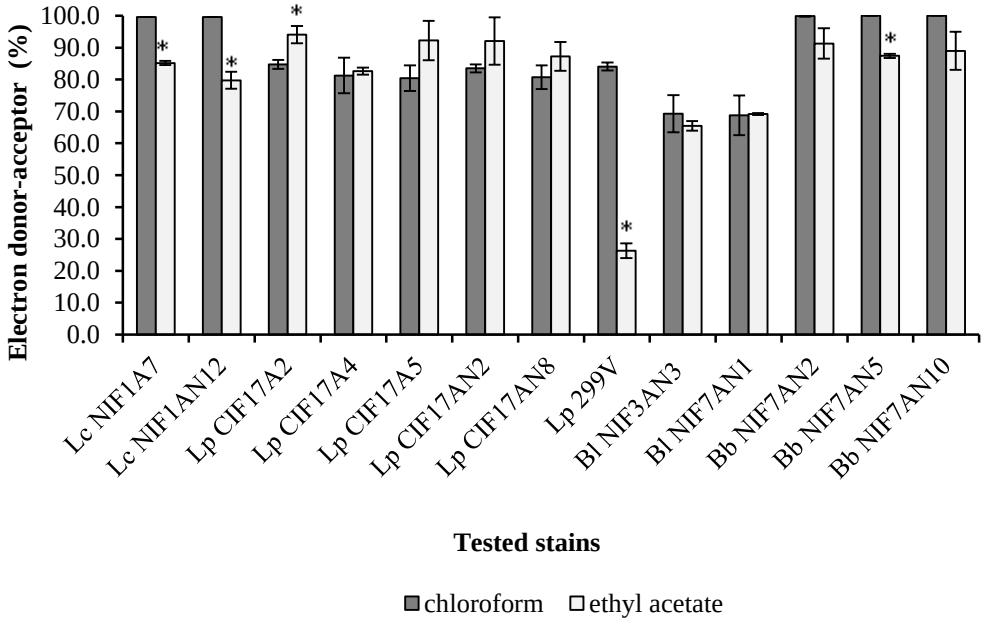


Figure 15. The percentages of adhesion to chloroform and ethyl acetate of mucin adhesive lactobacilli, bifidobacteria and *L. plantarum* 299V. * significant different ($P<0.05$) between two solvents.

4.5 Discussion

Adhesion to the intestinal mucosa was the first requirement for probiotic bacteria to exert its beneficial effects on host health (Muñoz-Provencio *et al*., 2009). Adhesion of bacteria to host mucin is regarded as major importance in contribution to permanent, or even transient, establishment and colonization of probiotic species in any environmental niche and also enhanced the ability to stimulate the immune and prevent the adhesion of pathogens (Collado *et al*., 2007c; Ouwehand *et al*., 1999; Ouwehand *et al*., 2001; Stamatova and Marounek, 2009; Wang *et al*., 2010b). Therefore, our lactobacilli and bifidobacteria isolated from infant feces were tested for their adhesion to porcine mucin. These human fecal isolate lactobacilli and bifidobacteria had adhesion ability to porcine mucin at various levels depending on species and strain. *L. casei* and *B. bifidum* showed adhesion ability better than well-known mucin adhesive *L. plantarum* 299V. Whereas, human isolate *L. platarum* and *B. longum* subsp. *longum* adhered to mucin in similar level with positive control.

The mucin adhesive lactobacilli and bifidobacteria still maintained adhesion ability after exposure to the simulated condition of stomach and small intestine. Interestingly, the intermediate mucin adhesive *L. plantarum*, such extreme condition had no impact on its adhesion ability. In contrast, the adhesion of high mucin adhesive *B. bifidum* was severely disturbed by low pH and bile salt condition. As well the adhesion of *L. brevis* PEL1 and *L. reuteri* ING1 was significantly ($P<0.05$) reduced by exposure to low pH (Ouwehand *et al*., 2001). Low pH and bile salt tolerant bacteria were not only important for bacterial survival in upper part of gastrointestinal tract but allow them to establish at the target site. Bile salt treated *B. bifidum* and *L.casei* significantly reduced their biding to immobilized mucin. It is possible that bile salt effect on hydrophobic component on the bacterial cell envelope: for example, lipoteichoic acids (Ouwehand *et al.,* 2001). Lipoteichoic acids have been observed to be important for the binding of *L. johnsonii* La1 to Caco-2 cells (Granato *et al*., 1999). In addition, the human gastrointestinal tract did not present only low acid and bile salt but also the protease enzyme like pepsin and pancreatin which may be affecting on cell surface organ involving in adhesion. The adhesion of *Bifidobacterium adolescentis* VTT E-001561 and *Bifidobacterium pseudolongum* ATCC 25526 was inhibited with pepsin at pH 2 and pancreatin (Crittenden *et al.,* 2001). Pepsin

treatment was found significantly ($P<0.05$) reduce the adhesion of *Lactobacillus brevis* PEL1, *Lactobacillus reuteri* INGI1, *Lactobacillus rhamnosus* E-800 and *Lactobacillus* GG to glycoprotein extracted from human faces.

Probiotic bacteria with adhesion ability may hinder pathogen association and invasion (Tsai *et al*., 2005). In this study the adhesive lactobacilli and bifidobacteria especially *Bifidobacterium* species have ability to inhibit adhesion of enteropathogens to mucin. However, competitive exclusion of these human isolate probiotic bacteria against pathogen was not correlated with their adhesion abiltiy.

Highly adhesive *L. casei* showed low competitive adhesion to mucin while intermediate mucin adhesive *L. plantarum* was not able to inhibit the adhesion of some pathogenic bacteria. In contrast, both high and intermediate mucin adhesive bifidobacteria exerted strongly competitive inhibition toward all tested pathogenic bacteria. The correlated results have previously been reported (Bibiloni *et al.,* 1999; Chouraqui *et al.,* 2004). These finding indicated that the inhibition against pathogen adhesion to mucin by probiotic bacteria appeared to depend bacterial genus. Bifidobacteria showed greather competitive inhibition than lactobacilli. This was similar with the result of Candela et al. (2005) who found that the non-adhesive *B. longum* E18 strain exerted strong displacement activity towards *Sal. enterica* serovar Typhimurium, *Yersinia enterocolitica* and *E. coli* H1040. In addition, there were other mechanisms invoving in competitive inhibition of probiotic bacteria. The inhibition could thus be related with the specific adhesions and receptors that probiotic and pathogen are competing for (Lee and Poung 2002). Another factor such as coagulation of both strains was proposed (Reid *et al.,* 1988; Gueimonde *et al.,* 2006). The coaggregation mechanisms, between pathogen and probiotic strains, could be involved in the reduction of pathogen adhesion to mucin. However, to improve the competitive adhesion of probiotic bacteria toward pathogenic bacteria, the combination of probiotic is one of alternative choice (Collado *et al*., 2007d).

Moreover, protein structure resembling at bacterial cell surface was also found to be responsible for adherence of lactobacilli to epithelial receptor, ileal mucin and biomaterial (Strus *et al*., 2001). S-layer protein is one of the surface associate proteins that have been found responsible for the adhesion of bacteria (Coconnier *et al*., 1992; Horie *et al*., 2002). S-layer proteins are composed of one (glyco) protein known as S-protein. These structures are macromolecular paracrytalline arrays that completely cover the bacterial cell

surface. S-layers are attached to the underlying cell wall by non-covalent bonds and usually may be dissociated and solubilized into protein monomers by chaotropic agents such as 5 M LiCl (Garrote *et al*., 2004). Chaotropic agent is substance which disrupts the structure and denatures macromolecule such as protein and nucleic acid by interfering intracellular interaction mediated by non-covalent force such as hydrogen bonds, vander Waals forces, hydrophobic interaction. Lithium chloride is a mild extraction agent. It extracted the S-layer protein from intact cell efficiently and selectively (Lortal *et al*., 1922; Messner *et al*., 1997). The lithium ion attacks the S-layer protein subunits, which are non-covalent linked to each other and disintegrates them into monomers by cation substitution. Therefore, lose of adhesion ability of probiotic bacteria after treating with 5 M LiCl could be indiated that S-layer protein involve in their adhesion.

To determine protein and S-layer protein involved in adhesion, mucin adhesive lactobacilli and bifidobacteria were trated with trypsin, proteinase K and LiCl. Proteinase K exhibits broad substrate specificity. It degrades many proteins in the native state enven in the presence of detergents. The predominant site of cleavage is the peptide bond adjacent to the carboxyl group of aliphatic and aromatic aminoacids with blocked alpha amino groups. Trypsin is a serine proteinase. Trypsin clevages peptide chains mainly at the carboxyl side of amino acids lysine or arginine.

After partially characterized by treating with proteinase K, trypsin and LiCl, we found that the adhesion capacity of high adhesive *B. bifidum* was significantly decreased. This indicated that the adhesion ability of these strong mucin adhesive bacteria was related to their cell surface protein. This result could be confirmed by reducing the adhesion ability of this bacterial species after exposure to proteolytic enzyme containing gastric and bile salt conditions.

Whereas, proteolytic enzyme and LiCl were not significant influence on the adhesion of intermediate mucin adhesive *L. plantarum* and *L. casei* one of mucin adhesive bacteria. Only some strains of these bacteria were affected from either trypsin or proteinase K. Although adhesion ability in all strains of *L. plantarum* was significantly decreased after LiCl treatment, more than half of their adhesion ability still maintained. This inferred that protein hardly or a bit involved in adhesion of *L. plantarum* and *L. casei*. The result could be confirmed by only a bit decrease in adhesion of these bacteria after exposure to acid and

bile solution containing proteolytic enzyme. It seems to be that there are other factors influences on mucin adhesion in these bacteria.

The physicochemical surface characteristics of bacteria are important in the first interactions that precede bacterial adhesion (Muñoz-Provencio *et al*., 2009). To determine bacterial cell surface hydrophobicity and electron acceptor-donor properties, the mucin adhesive bacteria were tested for their adhesion to apolar solvent, ethyl acetate and chloroform, respectively. We found the correlation between adhesion ability and cell surface hydrophobicity in *L. casei, B. bifidum* and *L. plantarum*. High mucin adhesive *L. casei* and *B. bifidum* showed strong cell surface hydrophobicity and electron donor properties while intermediate mucin adhesive *L. plantarum* exhibited moderate cell surface hydrophobicity and electron acceptor properties. From this result indicated that cell surface hydrophobicity maybe involved in adhesion of these bacteria to mucin. In addition, involvement of hydrophobicity and bacterial cell surface protein in adhesion of high mucin adhesive strains could be imply that protein presented on bacterial surface were hydrophobic. Many studies have shown that the presence of (glycol-) proteinaceous material at cell surface results in higher hydrophobicity (Pelletier *et al.,* 1997; Xu *et al.,* 2009). However, no correlation between cell surface hydrophobicity and adhesion to mucin was found in *B. longum* subsp. *longum* and *L. plantarum* 299V. These bacteria showed intermediate adhesion to mucin but cell surface characteristic exhibited weak hydrophobicity. Low cell surface hydrophobicity in mucs adhesive *L. plantarum* 299V has been reported (Muñoz-Provencio *et al*., 2009).

4.6 Conclusion

Our lactobacilli and bifidobacteria isolated from infant feces presented interesting probiotic properties by adhering to mucosal mucin. *B. bifidum* and *L. casei* were strong mucin adhesive probiotic bacteria whereas; *L. plantarum* and *B. longum* subsp. *longum* were intermediate mucin adhesive strains. Adhesion ability of these bacteria still maintain after passed through simulated condition in upper gastrointestinal tract. In addition, mucin adhesive lactobacilli and bifidobacteria were able to inhibit the adhesion of enteropathogens to mucin by competitive adhesion. The competitive inhibition activity did not correlate with adhesion ability of probiotic bacteria. Bifidobacteria was stronger competitive inhibitor than lactobacilli. Bacterial surface protein was extremely involved in

adhesion of high mucin adhesive *B. bifidum*. The physicochemical surface characteristic of this high mucin adhesive bacterium was strong hydrophobicity and electron donor properties.

CHAPTER 5

IN VITRO SCREENING OF PROBIOTIC LACTOBACILLI AND PREBIOTIC PLANT EXTRACTS TO SELECT EFFECTIVE SYNBIOTIC

5.1 Abstract

Ethanolic and water extracts from saba (*Musa sapientum* (Linn)) (Kluai Hin), gros michel (*Musa sapientum* (L.)) (Kluai Hom), okra *(Abelmochus esculentus* (L.) *Moench*) (Kra Jeab Khiew), mung bean (*Vigna radiate* (L.) R. Wilczek) (Tour Khiew) were evaluated for their prebiotic characteristics to support growth of 7 potential probiotic lactobacilli. Saba was the best plant extract to support the growth of 2 strains of *Lactobacillus casei* (NIF1A7and NIF1AN12) and 4 strains of *Lactobacillus plantarum* (CIF17A4, CIF17A5, CIF17AN2 and CIF17AN8), whereas gros michel was the best plant extract to support the growth of *L. plantarum* CIF17A2. These combinations were further evaluated for their ability to inhibit the growth of pathogenic bacteria in co-culture experiment. The combination of saba extract with either CIF17AN2 or CIF17AN8 was the most effective at inhibiting the growth of pathogenic bacteria by reducing the pathogenic number to undetectable within 24 h of co-cultivation time. Therefore, these combinations were selected and studied for their efficiency on major human gut microflora in mixed culture under un-controlled pH system. Both synbiotic formulae have not changed much in total bacteria (DAPI), eubacteria (Eub 338), lactobacilli (Lac 158) and bacteroides (Bac 303) count. While these combinations significantly increased bifidobacteria count. Significant decrease in clostridia (Chis 150) count was observed in the combination of saba extract and *L. plantarum* CIF17AN2 and also *L. plantarum* CIF17AN2 alone. In contrast, the clostridia count significantly increased in treatment with saba extract alone. Therefore saba extract did not specifically enhance growth of beneficial gut bacteria.

5.2 Introduction

Human gastrointestinal tract harbor a hundreds of different types of microorganisms including bacteria, fungi and protozoa (Allan *et al*., 1998; Grimoud *et al*., 2010), which have an enormous impact on host health. These microorganisms served as numerous important functions for its host through its metabolic activities and physiological regulation such as promotion of nutrient absorption, synthesis of bioactive compounds, improvement of intestinal barrier function, motility, resistance to pathogens or modulation of the immune system (Palmer *et al*., 2007; Grimoud *et al.*, 2010). Overall, a balanced gut microbiota composition confers benefits to the host, while microbiota imbalances are associated with metabolic and immune-mediated disorders (Laparra and Sanze, 2010). Alteration of the microbiota may cause some direct or indirect digestive pathology like infectious diseases and chronic inflammation, metabolic disorders or atopic diseased (Grimoud *et al*., 2010). One approach to health maintenance, disease control restore the intestinal ecosystem is the use of probiotic and prebiotic (Mandadzhieva *et al*., 2011). A probiotic is organism with a sufficient number of viable microorganisms altering the host's microbiota to produce beneficial health (Schrezenmeir and de Vrese, 2001). Most probiotic strains belong to *Lactobacillus* and *Bifidobacterium* genera, which are health promoting bacteria forming part of the balanced intestinal microflora. Prebiotics are non-digestible food ingredients, such as inulin and oligosaccharides that specifically stimulate development, activity or growth of beneficial intestinal bacteria (Young *et al.,* 2003; Geier *et al*., 2007). The main prebiotics used are fiber and carbohydrates, which are short polymers of glycosidic residues such as fructose in fructooligosaccharides or galactose in galactooligosaccharides. Naturally occurring prebiotic oligosaccharides are found in many sources such as plants (fructooligosaccharides, inulin, soybean oligosaccharides and xylooligosaccharide). The edible parts of plant which are commonly consumed in Thailand have been reported for their prebiotic components (Moongngarm *et al*., 2011; Wichienchot *et al*., 2011). Potentially, probiotic and prebiotic combinations (termed as synbiotics) may have benefits greater than either probiotic or prebiotic alone, because the prebiotic can enhance the growth, colonization, or activation of the probiotic species under nutrient limitation of human colon (Geier *et al*., 2007). Furrie *et al*. (2005) has been reported the capacity of synbiotic combination of *Bifidobacterium longum* and prebiotic to

reduce inflammation in patients with ulcerative colitis. The combination of lactitol and *Lactobacillus acidophilus* NCFM^{TM} were found to exhibit complementary beneficial effects on the colon microbial composition and activity (Mäkivuokko *et al*., 2010).

This study aimed to evaluate growth of 7 potential probiotic bacteria isolated from Thai infant feces in the presence of plant extracts prepared from saba (*Musa sapientum* (Linn)) (Kluai Hin), gros michel (*Musa sapientum* (L.)) (Kluai Hom), okra *(Abelmochus esculentus* (L.) *Moench*) (Kra Jeab Khiew) and mung bean (*Vigna radiate* (L.) R. Wilczek) (Tour Khiew). The antimicrobial activity of the proper combination of plant extract and the selected probiotic against Gram-positive and Gram-negative pathogenic bacteria was determined in co-cultivation system. The synbiotic combination of plant extract and the selected probiotic bacteria was applied in mixed culture of fecal microflora to investigate change of major gut microflora population.

5.3 Materials and methods

5.3.1 Preparation of plant extract

Plant materials were obtained from local market in Songkhla, Thailand, including saba (*Musa sapientum* (Linn)) (Kluai Hin), gros michel (*Musa sapientum* (L.)) (Kluai Hom), okra *(Abelmochus esculentus* (L.) *Moench*) (Kra Jeab Khiew), mung bean (*Vigna radiate* (L.) R. Wilczek) (Tour Khiew). Saba, gros michel and mung bean were extracted in ethanol (50% V/V) and okra was extracted in water. The crude extracts were purified by precipitating twice in cool ethanol (80%). The purified extracts were freeze dried to obtain dry powder and measured total and reducing sugar content by phenol-sulfuric method (Fox and Robyt, 1991) and dinitrosalicylic acid (DNS) method (Robertson *et al.*, 2001), respectively with glucose as a standard.

5.3.2 Bacterial strains

The 7 strains of lactobacilli used in this study including *Lactobacillis casei* NIF1A7, *Lactobacillus casei* NIF1AN12, *Lactobacillus plantarum* CIF17A2, *Lactobacillus plantarum* CIF17A4, *Lactobacillus plantarum* CIF17A5, *Lactobacillus plantarum* CIF17AN2 and *Lactobacillis plantarum* CIF17AN8. All lactobacilli strains were isolated from infant feces and already tested for their probiotic properties. The probiotic bacteria

were routinely cultured in MRS medium and incubated at 37 °C for 24 h. *Escherichia coli* TISTR 780 and *Staphylococcus aureus* TISTR 1466 were from Microbiological Resources Centre (MIRCEN), Thailand. *Escherichia coli* O157: H7 DMST 12743 was from the Department of Medical Science, Ministry of Public Health, Thailand. *Shigella sonei, Shigella flexneri, Salmonella enterica* subsp. *enterica* serovar Typhimurium SA2093 and *Salmonella* Paratyphi A were from the Microbiological Laboratory of Songklanakarind Hospital, Songkhla, Thailand. All pathogens were grown in Muller Hinton broth (MHB; Himedia, Mumbai, India) at 37 °C for 24 h.

5.3.3 Effect of prebiotics on the growth of the potential probiotic bacteria

A minimal medium containing (g l^{-1}); peptone water 2, yeast extract 2, NaCl 0.1, K_2HPO_4 0.04, KH_2PO_4 0.04, $MgSO_4 \cdot 7H_2O$ 0.01, $CaCl_2 \cdot 6H_2O$ 0.01, $NaHCO_3$ 2, hemin 0.05 (dissolved in a few drops of 1 mol/l NaOH), cysteine·HCl 0.5, bile salts 0.5, Tween 80 2 and 10 μl vitamin K was added with 1% (based on total sugar content) of each prebiotic extract. The medium was adjusted to pH 7.0 using 1 M HCl (Rycroft *et al.*, 2001). Overnight culture of probiotic bacteria were centrifuged and washed with phosphate buffer saline (PBS 7.2) and then resuspended with minimal medium for using as inoculums. The 10% of probiotic inoculum was inoculated to the minimal medium containing different prebiotic extracts. The tested tubes were incubated at 37°C for 24 h. Samples were taken for enumeration of probiotic bacteria on MRS agar, pH changing, carbohydrate utilization, and antibacterial activity as the followings:

5.3.3.1 Growth of probiotic in minimal medium containing prebiotic extract

The samples were serially diluted. One hundred microliter of appropriate dilutions was placed on MRS agar. Plates were incubated at 37°C for 24-48 h.

5.3.3.2 Carbohydrate utilization

Carbohydrate utilization was determined in cell free culture supernatant by phenol-sulfuric acid method described by Fox and Robyt (1991). To obtain cell free culture supernatant, the samples were centrifuged at 10,000 rpm for 10 min. The supernatant (25 μl) were added in triplicate into a 96-well general assay plate followed by 25 μl of 5% (w/v) phenol. In addition to the test samples, standards of known glucose concentration were placed triplicate in each plate. The standards were 0, 20, 40, 60, 80, 100, 120, 140,

160, 180, 200, 220 and 240 mg ml^{-1} of glucose (distilled water was used as blank). After all the samples were loaded, the plate was mixed for 30 s. The plate was placed on ice, and 125 µl of concentrated H_2SO_4 was added to each well. The plate was mixed for 30 s. The plate was sealed in a plastic zipper bag and warmed in a water bath at 80°C for 30 min. To measure the remaining total sugar the plate was read with microtiter plate reader at 492 nm. The remainder glucose concentration was determined by comparing with standard glucose.

5.3.3.3 Antibacterial activity

Broth microdilution assay in 96-well plates was used to determine antimicrobial activity. The samples were centrifuged at 10,000 rpm, 10 min, at 4 °C. A series two-fold dilution of the cell free supernatant (100 µl) in Muller Hinton broth (MHB) (100 µl) were prepared in triplicate. Bacterial pathogens including *E. coli* TISTR 780, *S. aureus* TISTR 1466, *Shi. sonnei*, *Shi. flexneri*, *Sal.* Paratyphi A, and *Sal. enterica* subsp. *enterica* serovar Typhimurium SA2093 were cultivated in MHB at 37°C for 18 h. The cultures were diluted with MHB to achieve 1×10^6 CFU ml^{-1}. Ten microliter of each pathogen was then added to each well to obtain a final concentration of about 10^5 CFU ml^{-1}. Plates were incubated at 37°C for 24 h. The undiluted supernatant and MHB inoculated with each pathogen were used as positive and negative control, respectively. The inhibition activity was expressed as an arbitrary unit (AU) per milliliter. An arbitrary unit was defined as the reciprocal of the highest dilution which did not give a visible growth of bacteria (Millette *et al*., 2007). The AU ml^{-1} was calculated according to (1000/100)*D*, whereas *D* was the dilution factor.

5.3.4 Antimicrobial activity of probiotic bacteria toward pathogenic bacteria in medium containing prebiotic extract under co-culture system

Lactobacillus casei NIF1A7, *L. casei* NIF7AN12, *L. plantarum* NIF17A4, *L. plantarum* NIF17A5, *L. plantarum* CIF17AN2 and *L. plantarum* CIF17AN8 were cultured in medium contained saba extract. *L. plantarum* CIF17A2 was cultured in medium containing gros michel. Minimal medium supplemented with 1% of each prebiotic extract was prepared in 50 ml injection vial. *E. coli* TISTR 780, *S. aureus* TISTR 1466 and *Sal*. Typhimurium SA2093 were used as representative Gram-positive and Gram-negative pathogenic bacteria. Overnight cultures of each probiotic strain and each enteropathogenic

strain were centrifuged and inoculated into medium to give final concentration of 10^5 CFU ml^{-1} and then co-cultivation was operated at 37 °C under anaerobic condition. The survival of pathogenic bacteria was counted by culturing on MH agar containing bromocresol purple (probiotic bacteria were not able to grow in this medium) and the growth of probiotic bacteria were quantified by culturing on MRS agar then plates were incubated for 24-48 h at 37 °C under appropriate conditions. Each experiment was repeated in triplicate. The culture of each test strain was used as control.

5.3.5 Effect of probiotic, prebiotic and synbiotic on dominant gut microflora in small scale un-controlled pH batch culture fermentation

L. plantarum CIF17AN2 and *L. plantarum* CIF17AN8 expressed effectively antimicrobial activity against pathogenic bacteria in co-cultivation assay therefore these 2 strains were selected and combined with prebiotic extract, then studied for their efficacy on dominant human fecal microbiota. Small scale batch culture fermentation was set up in 50 ml serum vial. The vials were filled with 40 or 45 ml of basal medium then flushed with O_2-free N_2 to provide anaerobic condition and sealed with rubber stopper and secured by aluminum cap. The medium contained, per liter, 2 g of peptone water, 2 g of yeast extract, 0.1 g of NaCl, 0.04 g of K_2HPO_4, 0.01 g of $MgSO_4 \cdot 7H_2O$, 0.01 g of $CaCl_2 \cdot 6H_2O$, 2 g of $NaHCO_3$, 0.005 g of hemin, 0.0 5 g of L-cysteine hydrochloride, 0.5 g of bile salts, 2 ml of tween 80, 10 µl of vitamin K and 4 ml of 0.025% (w/v) resazurin solution. Stock carbohydrate (10%) was prepared by dissolving saba extract in sterile basal medium and then 5 ml was added into each vial to give final concentration of 1%. Fecal slurry (10 % w/v) was prepared using freshly stool samples from 3 healthy donors (who had not taken antibiotics for 3 months). The fecal samples were diluted with 0.1 mol l^{-1} phosphate buffer (pH 7.2); this was mixed in a stomacher for 2 min. The 5 ml of fecal slurry was injected into each serum vial. Overnight culture of *L. plantarum* CIF17AN2 and *L. plantarum* CIF17AN8 were centrifuged and resuspended in basal medium. After that, cell suspension was added into vial containing basal medium to give final concentration of 10^7 CFU ml^{-1}. The treatment was set as following; fecal slurry only (NC), *L. plantarum* CIF17AN2 only, *L. plantarum* CIF17AN8 only, saba extract only, *L. plantarum* CIF17AN2+saba extract and *L. plantarum* CIF17AN8+saba extract. After injection, the vials were incubated at 37 °C

and the samples were taken at time 0 h, 12 h, 24 h for enumeration of bacteria by fluorescent *in situ* hybridization (FISH). Each treatment was performed in triplicate.

5.3.5.1 Enumeration of bacteria using fluorescent *in situ* hybridization (FISH) technique

FISH technique was performed as described by Martin-Peláez et al. (2008). Samples taken from batch culture (375 µl) were added to 1.125 ml of 4% (w/v) filtered paraformaldehyde (chilled), mixed and stored at 4 °C overnight to fix the cell. The fixed cells were then centrifuged at 12,000 rpm (Eppendorf, Germany) for 5 minutes and washed twice with cool filtered sterilize 0.1 mol l^{-1} PBS, pH 7.2 and resuspended with 150 µl of PBS. After that, ethanol (150 µl) was added then mixed throughout. The fixed samples were stored at -20 °C at least 1 h or until further analysis (within 3 months). The fixed cells were diluted to obtain appropriate dilution and then 20 µl of suitable dilutions were placed onto the well of Teflon- and Poly-L-lysine coated slides. The slides were placed on slide dryer (46-50 °C, 15 min) followed by permeabilization in alcohol series (50, 80 and 90% ethanol) for 3 minutes of each concentration to allow the penetration of probe and finally dried on slide dryer. Lysozyme treatment was need for *Lactobacillus*/*Enterococcus* spp. before dehydration. Pre-warmed hybridization buffer was mixed with 5 µl of genus-specific 16S rRNA target oligonucleotide probes (50 ng μl^{-1}). Fluorescent dye Cy3 labeled oligonucleotide probe specific for *Bifidobacterium, Bacteroides, Lactobacillus/ Enterococcus* spp., *Clostridium histolyticum* group, and *Eubacterium* group were Bif 164 (5'-CATCCGGCATTACCACCC-3′) Bac 303 (5′-CCAATGTGGG GGACCTT-3′), Lab 158 (5′-GGTATTAGCAYCTTCCA-3′), chis 150 (5′-TTATGCGGTATTAATAT(C/T) CCTTT-3′), Eub 338 (5′- GCTGCCTCCCGTAGGAGT -3′), respectively (Rycroft *et al.*, 2001; Rochet, Rigottier-Gois *et al*., 2004; Al-Tamimi *et al*., 2006; Mandalari *et al*., 2007) and nucleotide target 4′,6-diamidino-2-phenylindole (DAPI) (Sigma, Sigapore) dye was used for total bacterial counts. The probe solutions (50 µl) were added onto each well, and allowed to hybridize in hybridization oven (Boekel Scientific) for 4 h at 46 °C for Eub 338 and Bac 303 probes; 50 °C for Lac 158, Bif 164 and Chis 150 probes. After hybridization, the slides were washed with pre-warmed washing buffer, (0.9 mmol l^{-1} NaCl, 20 mmol l^{-1} Tris-HCl, pH 7.2) containing 20 µL DAPI solution (50 ng μl^{-1}) for 15 min at 48 or 50 °C depending on the probe. The slide was dipped into cold distilled water then quick drying.

Five microliters of antifade (Fluka, Singapore) were then added onto slide well and cover by cover slide before visualization with fluorescence microscope (Nikon Eclipse 80i, USA). The DAPI stained cells were examined under UV light and hybridized cells viewed using a Cy3 filter. A minimum of 15 fields, each containing 10-100 cells, was counted for each well.

5.4 Results

5.4.1 Growth of probiotic bacteria in minimal medium containing prebiotic extract

This step was performed to screen prebiotic extracts, which can enhance growth of the selected probiotic strains. The partial purified prebiotic extracts of mung bean, saba, gros michel, and okra were used as a carbon source in this study. The commercial prebiotic including FOS, inulin, GOS and glucose were used as control. Saba extract was the best prebiotic for *L. casei* NIF1A7, *L. casei* NIF1AN12, *L. plantarum* CIF17A4, *L. plantarum* CIF17A5, *L. plantarum* CIF17AN2 and CIF17AN8 by increasing their number 3.32, 3.09, 3.44, 3.16, 3.53, and 3.47 CFU ml^{-1}, respectively within 24 h (Figure 16A, 16B, 16D, 16E, 16F and 16G). Growth of these probiotic strains in medium added with saba extract was significantly greater than glucose as carbon source ($p<0.05$). Whereas, gros michel was the best carbon source for *L. plantarum* CIF17A2 by increasing its number 2.21 log CFU ml^{-1} which was significantly greater than glucose ($p<0.05$) as shown in Figure 16C. Okra, mung bean and GOS moderately supported the growth of probiotic bacteria. These ethanolic plant extract supported the growth of human isolate probiotic bacteria at same level as glucose. Five out of seven probiotic strains were able to utilize FOS as a carbon source. The growth of these strains was significantly higher than negative control (minimal medium without any carbon source). However, the selected probiotic bacteria in this study could not utilize inulin as a carbon sources.

Carbohydrate utilization in these 7 selected probiotic bacteria was shown in Table 17. Probiotic bacteria were able to utilize saba, okra, mung bean, gros michel, GOS and FOS as carbon source. However, all tested probiotic bacteria showed preferences for monosaccharide (glucose) as a carbon sources. Probiotic bacteria showed different preference for the types of oligosaccharides presence in each plant extract. The highest

carbohydrate consumption of probiotic bacteria was found in the media containing the extract of saba and gros michel. Carbohydrate utilization of saba and gros michel were more than 78% and 79% respectively. The moderate consumption of probiotic bacteria was found in medium containing the extract of okra, mung bean and commercial GOS. More than 50% of carbohydrate content in these three carbon sources was utilized by tested probiotic bacteria. Low carbohydrate consumption was found in medium contained commercial prebiotic including inulin and FOS. Carbohydrate consumption of probitic bacteria for these commercial prebiotic was less than 25%. Probiotic growth and carbohydrate utilization were correlated with changing in pH (Table 18). The highest decrease in pH was found in supernatants of the culture in the medium containing extract from okra, gros michel, saba and glucose (control). No changing in pH was found in the supernatant of culture medium containing inulin and FOS.

5.4.2 Inhibitory effect of probiotic against foodborne pathogens in the presence of prebiotic extracts

Enhancement of antimicrobial activity of probiotic bacteria is one of prebiotic properties. Inhibitory activity of all 7 strains probiotic bacteria against *E. coli* TISTR, *S. aureus* TISTR 1466, 780, *Sal*. Typhimurium SA2093, *Sal.* Paratyphi A, *Shi. sonei* and *Shi. flexneri* were shown in Table 19, Table 20, Table 21, Table 22, Table 23 and Table 24. Supernatants from probiotic cultured in the medium added with extracts from saba, gros michel and glucose as control exhibited antimicrobial activity against all foodborne pathogens. The antimicrobial activity of these supernatants was 10-20 AU ml^{-1}. In addition, the supernatant of *L. plantarum* CIF17AN2 cultured in the medium supplemented with GOS was also able to inhibit the growth of all pathogenic bacteria with antimicrobial activity of 10 AU ml^{-1}. However, no antimicrobial activity was found on food borne pathogens for supernatant of probiotic culture in medium containing inulin, FOS, and the extract of mung bean and okra.

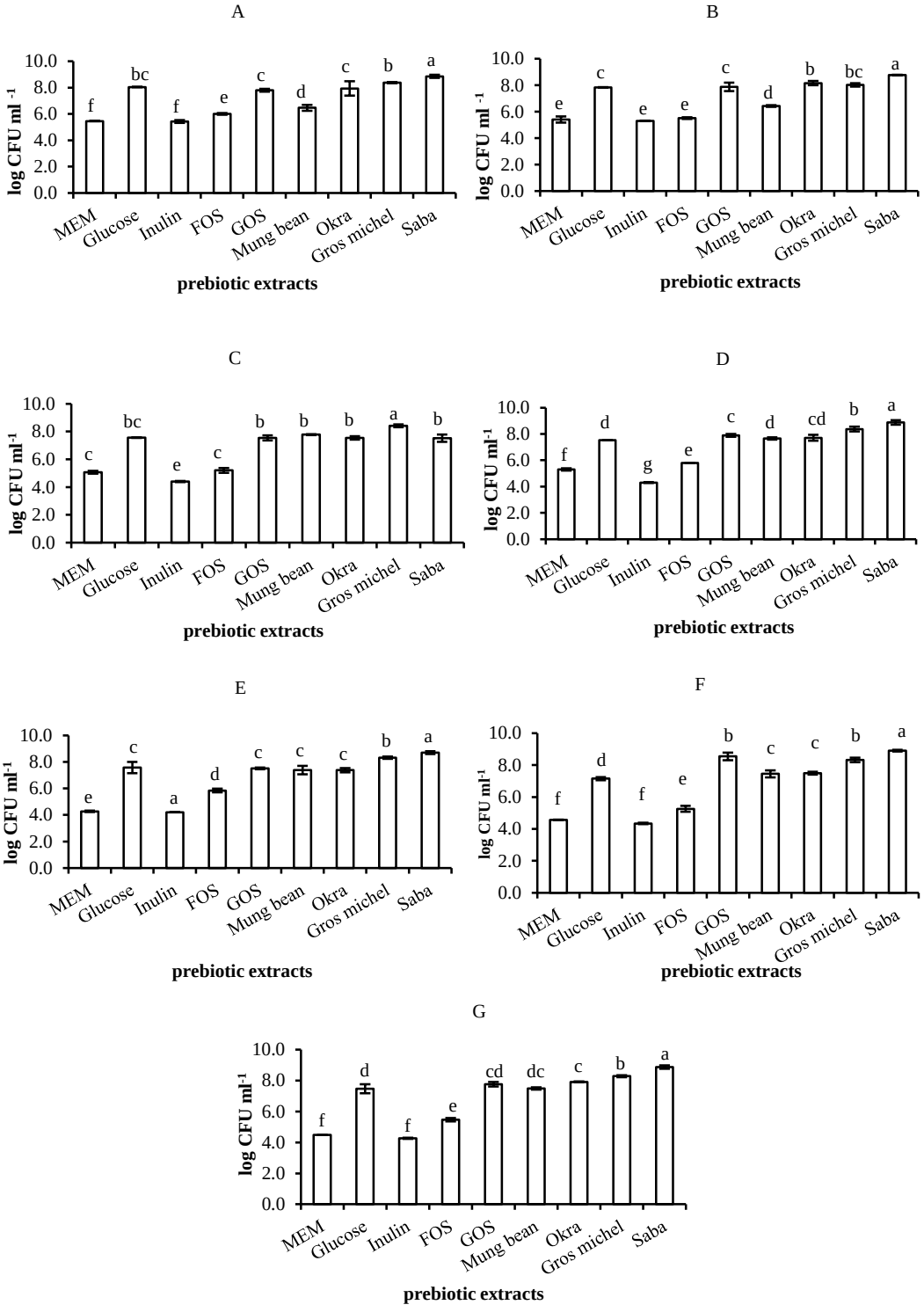


Figure 16. Growth of *L. casei* NIF1A7 (A), *L. casei* NIF1AN12 (B), *L. plantarum* CIF17A2 (C), *L. plantarum* CIF17A4 (D), *L. plantarum* CIF17A5 (E), *L. plantarum* CIF17AN2 (F), and *L. plantarum* CIF17AN8 (G) in minimal medium containing inulin, FOS, GOS, mung bean, okra, gros michel, saba and glucose as carbon sources.

Table 17. Carbohydrate utilization (%) of the selected probiotic bacteria grown in minimal medium containing glucose, inulin, FOS, GOS, mungbean, okra, gros michel and saba.

LAB strains	Carbohydrate utilization (%)							
	Glucose	Inulin	FOS	GOS	Mung bean	Okra	Gros michel	Saba
L casei NIF1A7	74.62	15.24	25.05	60.45	59.40	72.2	79.40	82.2
L. casei NIF1AN12	80.62	11.84	15.78	63.34	59.28	77.12	79.28	81.32
L. plantarum CIF17A2	94.82	7.12	17.04	63.80	68.93	69.23	88.93	79.45
L. plantarum CIF17A4	79.65	8.69	17.82	65.96	65.19	64.16	85.19	80.06
L. plantarum CIF17A5	74.62	5.85	16.21	65.96	66.55	66.13	86.55	79.54
L. plantarum CIF17AN2	84.42	6.71	13.16	76.52	66.92	67.24	86.92	78.31
L. plantarum CIF17AN8	88.68	5.98	15.19	65.75	66.88	68.97	86.88	79.62

Table 18. pH values of the overnight culture broth of probiotics grown in minimal medium containing glucose, inulin, FOS, GOS, saba, okra, mungbean, okra, gros michel and saba.

LAB strains	pH of supernatant							
	Glucose	Inulin	FOS	GOS	Mung bean	Okra	Gros michel	Saba
L. casei NIF1A7	3.44	6.45	6.11	4.10	6.59	3.89	3.84	4.26
L. casei NIF1AN12	3.39	6.46	6.65	4.15	6.19	3.88	3.95	4.25
L. plantarum CIF17A2	3.40	6.42	6.03	4.70	6.98	3.89	3.91	4.39
L. plantarum CIF17A4	3.35	6.76	7.01	5.37	6.76	3.94	3.86	4.43
L. plantarum CIF17A5	3.59	6.85	6.98	5.37	6.91	3.90	4.11	4.42
L. plantarum CIF17AN2	3.34	6.90	6.99	5.49	6.09	3.93	3.92	4.44
L. plantarum CIF17AN8	3.35	7.00	6.92	5.68	6.96	3.91	3.99	4.44

Table 19. Antimicrobial activity of the supernatant from LAB cultivated in minimal medium containing various prebiotic extracts against *E. coli* TISTR 780.

strains	Antimicrobial activity (AU ml^{-1}) *E. coli* TISTR 780							
	Glucose	Inulin	FOS	GOS	Saba	Okra	Mung bean	Grosmichel
L. casei NIF1A7	20	0	0	0	10	0	0	10
L. casei NIF1AN12	10	0	0	0	10	0	0	10
L. plantarum CIF17A2	10	0	0	0	10	0	0	10
L. plantarum CIF17A4	10	0	0	0	10	0	0	10
L. plantarum CIF17A5	10	0	0	0	10	0	0	10
L. plantarum CIF17AN2	10	0	0	10	20	0	0	10
L. plantarum CIF17AN8	10	0	0	0	20	0	0	10

Table 20. Antimicrobial activity of the supernatant from LAB cultivated in minimal medium containing various prebiotic extracts against *S. aureus* TISTR 1446.

Strains	Antimicrobial activity (AU ml^{-1}) *S. aureus* TISTR 1466							
	Glucose	Inulin	FOS	GOS	Saba	Okra	Mung bean	Gros michel
L. casei NIF1A7	20	0	0	0	10	0	0	10
L. casei NIF1AN12	10	0	0	0	10	0	0	10
L. plantarum CIF17A2	10	0	0	0	10	0	0	10
L. plantarum CIF17A4	10	0	0	0	10	0	0	10
L. plantarum CIF17A5	10	0	0	0	10	0	0	10
L. plantarum CIF17AN2	10	0	0	10	20	0	0	10
L. plantarum CIF17AN8	10	0	0	0	20	0	0	10

Table 21. Antimicrobial activity of the supernatant from LAB cultivated in minimal medium containing various prebiotic extracts against *Sal.* Typhimurium SA2093.

Strains	Antimicrobial activity (AU ml^{-1}) *Sal.* Typhimurium SA2093							
	Glucose	Inulin	FOS	GOS	Saba	Okra	Mung bean	Gros michel
L. casei NIF1A7	20	0	0	0	10	0	0	10
L. casei NIF1AN12	10	0	0	0	10	0	0	10
L. plantarum CIF17A2	10	0	0	0	10	0	0	10
L. plantarum CIF17A4	10	0	0	0	10	0	0	10
L. plantarum CIF17A5	10	0	0	0	10	0	0	10
L. plantarum CIF17AN2	20	0	0	10	20	0	0	10
L. plantarum CIF17AN8	20	0	0	0	20	0	0	10

Table 22. Antimicrobial activity of the supernatant from LAB cultivated in minimal medium containing various prebiotic extracts against *Sal.* Paratyphi A.

strains	Antimicrobial activity (AU ml^{-1}) *Sal*. Paratyphi A							
	Glucose	Inulin	FOS	GOS	Saba	Okra	Mung bean	Grosmichel
L. casei NIF1A7	20	0	0	0	10	0	0	10
L. casei NIF1AN12	10	0	0	0	10	0	0	10
L. plantarum CIF17A2	10	0	0	0	10	0	0	10
L. plantarum CIF17A4	10	0	0	0	10	0	0	10
L. plantarum CIF17A5	20	0	0	0	10	0	0	10
L. plantarum CIF17AN2	20	0	0	10	10	0	0	10
L. plantarum CIF17AN8	20	0	0	0	10	0	0	10

Table 23. Antimicrobial activity of the supernatant from LAB cultivated in minimal medium containing various prebiotic extracts against *Shi. sonei*.

Strains	Antimicrobial activity (AU ml^{-1}) *Shi. sonei*							
	Glucose	Inulin	FOS	GOS	Saba	Okra	Mung bean	Grosmichel
L. casei NIF1A7	20	0	0	0	10	0	0	10
L. casei NIF1AN12	10	0	0	0	10	0	0	10
L. plantarum CIF17A2	10	0	0	0	10	0	0	10
L. plantarum CIF17A4	10	0	0	0	10	0	0	10
L. plantarum CIF17A5	10	0	0	0	10	0	0	10
L. plantarum CIF17AN2	20	0	0	10	10	0	0	10
L. plantarum CIF17AN8	20	0	0	0	10	0	0	10

Table 24. Antimicrobial activity of the supernatant from LAB cultivated in minimal medium containing various prebiotic extracts against *Shi. flexneri.*

strains	Antimicrobial activity (AU ml^{-1}) *Shi. flexneri*							
	Glucose	Inulin	FOS	GOS	Saba	Okra	Mung bean	Grosmichel
L. casei NIF1A7	20	0	0	0	10	0	0	10
L. casei NIF1AN12	10	0	0	0	10	0	0	10
L. plantarum CIF17A2	10	0	0	0	10	0	0	10
L. plantarum CIF17A4	10	0	0	0	10	0	0	10
L. plantarum CIF17A5	10	0	0	0	10	0	0	10
L. plantarum CIF17AN2	20	0	0	10	10	0	0	10
L. plantarum CIF17AN8	20	0	0	0	10	0	0	10

5.4.3 Antimicrobial activity of the selected probiotics against foodborne pathogens in co-cultivation study of pure culture system

The ability of probiotic bacteria to inhibit the growth of pathogens was tested by co-cultivation of probiotic with each pathogen in minimal medium containing 1% the selected prebiotic extract. The saba extract that enhanced growth and antimicrobial activity of *L. casei* NIF1A7, *L. casei* NIF1AN12, *L. plantarum* CIF17A4, *L. plantarum* CIF17A5, *L. plantarum* CIF17AN2, *L. plantarum* CIF17AN8 was selected and used as carbon source for these probiotic bacteria. Gros michel was used as sole carbon source for *L. plantarum* CIF17A2. *E. coli* TISTR 780, *S. aureus* TISTR 466 and *Sal.* Typhimurium SA2093 were the representative pathogenic bacteria. Co-cultivation of probiotic bacteria and pathogen in medium containing glucose (1%) as carbon source was used as control.

In co-culture study, *S. aureus* TISTR 1466, *E. coli* TISTR 780 and *Sal.* Typhimurium SA2093 inhibition was observed in all treatments which prebiotic extract was used as carbon source (Figure 17, 18 and 19). *L. plantarum* CIF17AN2 and CIF17AN8 combined with saba extract showed the most effective inhibition against all three tested pathogens. Complete inhibition of pathogens was detected after 24 h of incubation. The complete inhibitory effect of another strain was found at 48 h of co-cultivation period. In contrast, some combinations only reduced a certain number of the pathogens. More than 2 log CFU ml^{-1} of *S. aureus* TISTR 1466 still survived at 72 h incubation in the presence of *L. plantarum* CIF17A4 and CIF17A5. Low antimicrobial activity of probiotic bacteria was found when glucose was use as a carbon source. Viable cell of pathogens was maintained until 72 h of incubation time. Only the combination of *L. casei* NIF1AN12, *L. plantarum* CIF17A4 and CIF17A5 with glucose showed antimicrobial activity against *Sal*. Typhimurium SA2093 with complete inhibition.

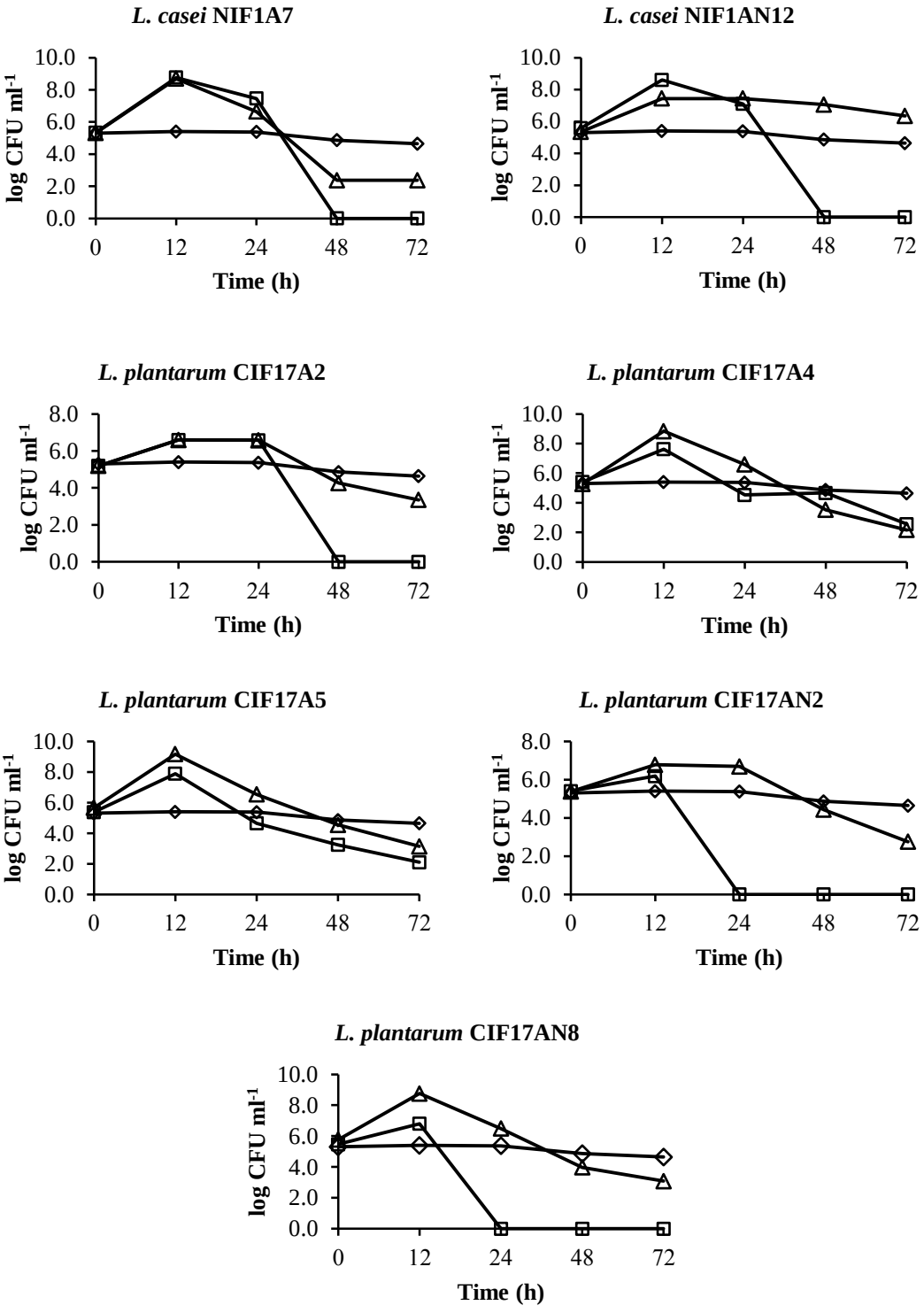


Figure 17. Antimicrobial activity of probiotic bacteria against *S. aureus* TISTR 1466 in co-cultivation (pathogen alone; ◇, pathogen in co-culture with saba extract as carbon source; □, pathogen in co-culture with glucose as carbon source; Δ.

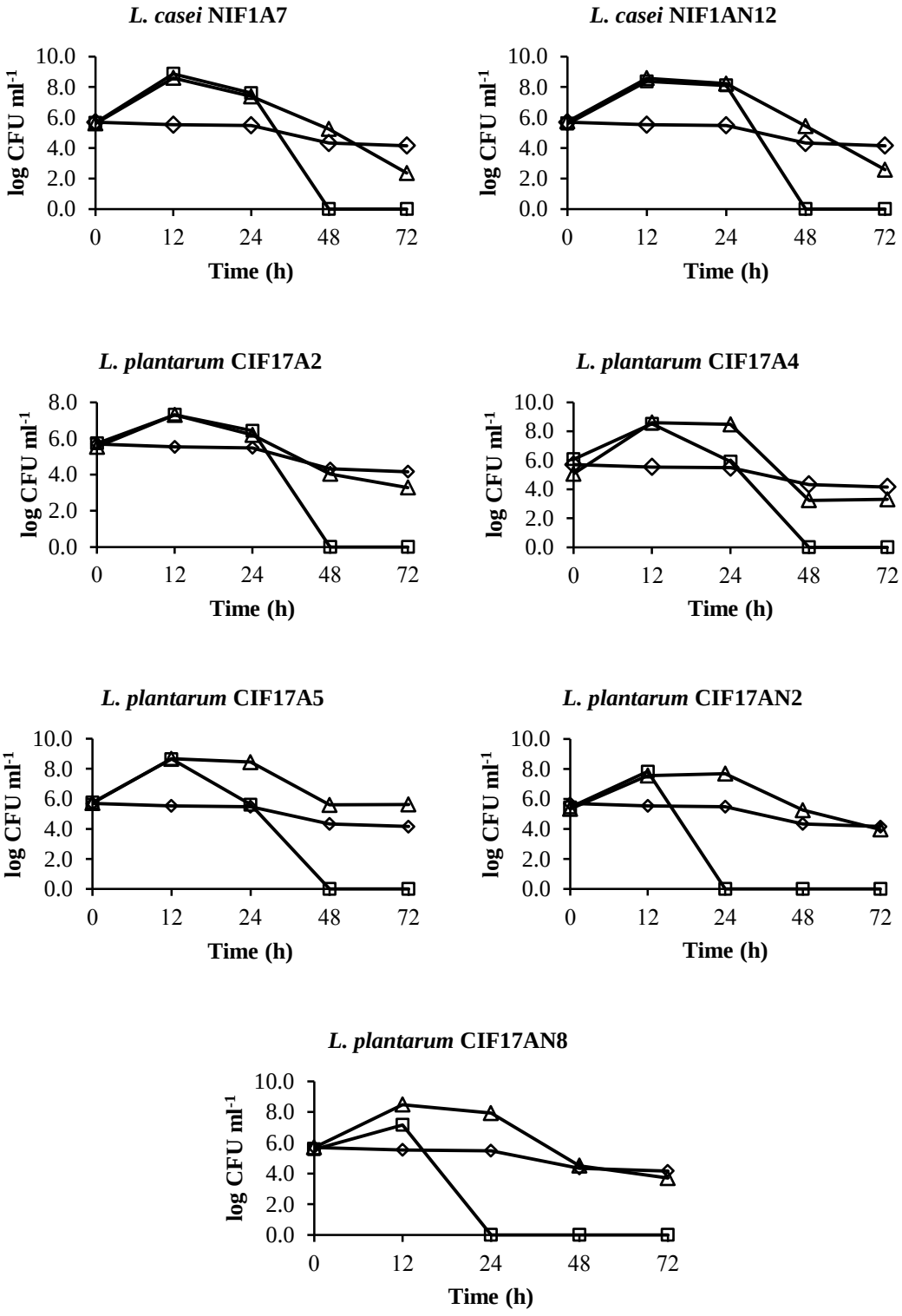


Figure 18. Antimicrobial activity of probiotic bacteria against *E. coli* TISTR 780 in co-cultivation (pathogen alone; ◇, pathogen in co-culture with saba extract as carbon source; □, pathogen in co-culture with glucose as carbon source; Δ.

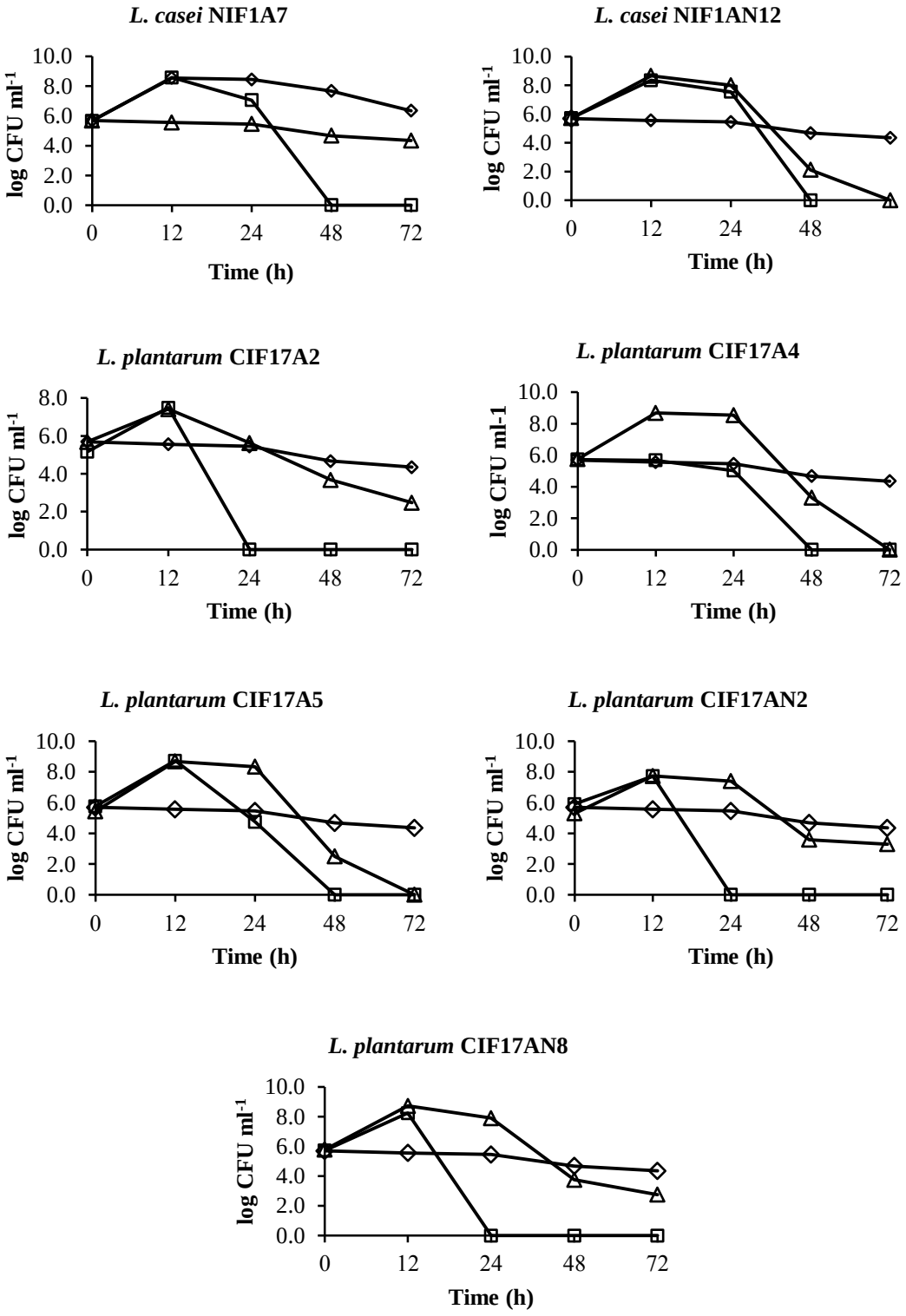


Figure 19. Antimicrobial activity of probiotic bacteria against *Sal.*Typhimurium SA2093 in co-cultivation (pathogen alone; ◇, pathogen in co-culture with saba extract as carbon source; □, pathogen in co-culture with glucose as carbon source; Δ.

5.4.4. Effect of *L. plantarum* CIF17AN2 and *L. plantarum* CIF17AN8 with and without combination of saba extract on dominant fecal microflora in the mixed culture system under anaerobic condition

Small scale batch culture fermentation was operated to investigate the effect of *L. plantarum* CIF17AN2 and *L. plantarum* CIF17AN8 with and without combination of saba extract on the major group of human fecal microflora. The results are shown in Figure 20, *L. plantarum* CIF17AN2 and *L. plantarum* CIF17AN8 with and without combination of saba extract and also saba extract alone trended to exert beneficial effect on dominant gut microflora by increasing the total bacteria (DAPI), eubacteria (Eub 338) and lactobacilli/enterococci (Lac 158) count. The number of these bacteria in supplemented treatment was higher than negative control which was a minimal medium containing only fecal sample. However, there were no significant differences among supplementation groups. The bacteroides count was stable during fermentation period in all treatments. There was no significant difference in bacteriodes count between supplementation group and control. Whereas, the 2 combinations of *L. plantarum* CIF17AN2+saba extract and *L. plantarum* CIF17AN8+saba extract and saba extract alone significantly increased bifidobacteria count. Bifidobacteria number in these treatments was obviously higher than other treatment. However, there was no significant different between two combinations. At 12 h of fermentation, decreas in clostridia count was found in all supplemented treatments, especially in treatment presented of *L. plantarum* CIF17AN2. Significant decrease in clostridia number was observed in experiment carried out with this strain both with and without saba extract combination. The dramatic decrease in clostridia number in these treatments was investigated when the incubation period reached to 24 h. Unfortunately, at 24 h of fermentation, increase in clostridia count was observed when saba extract was presented alone.

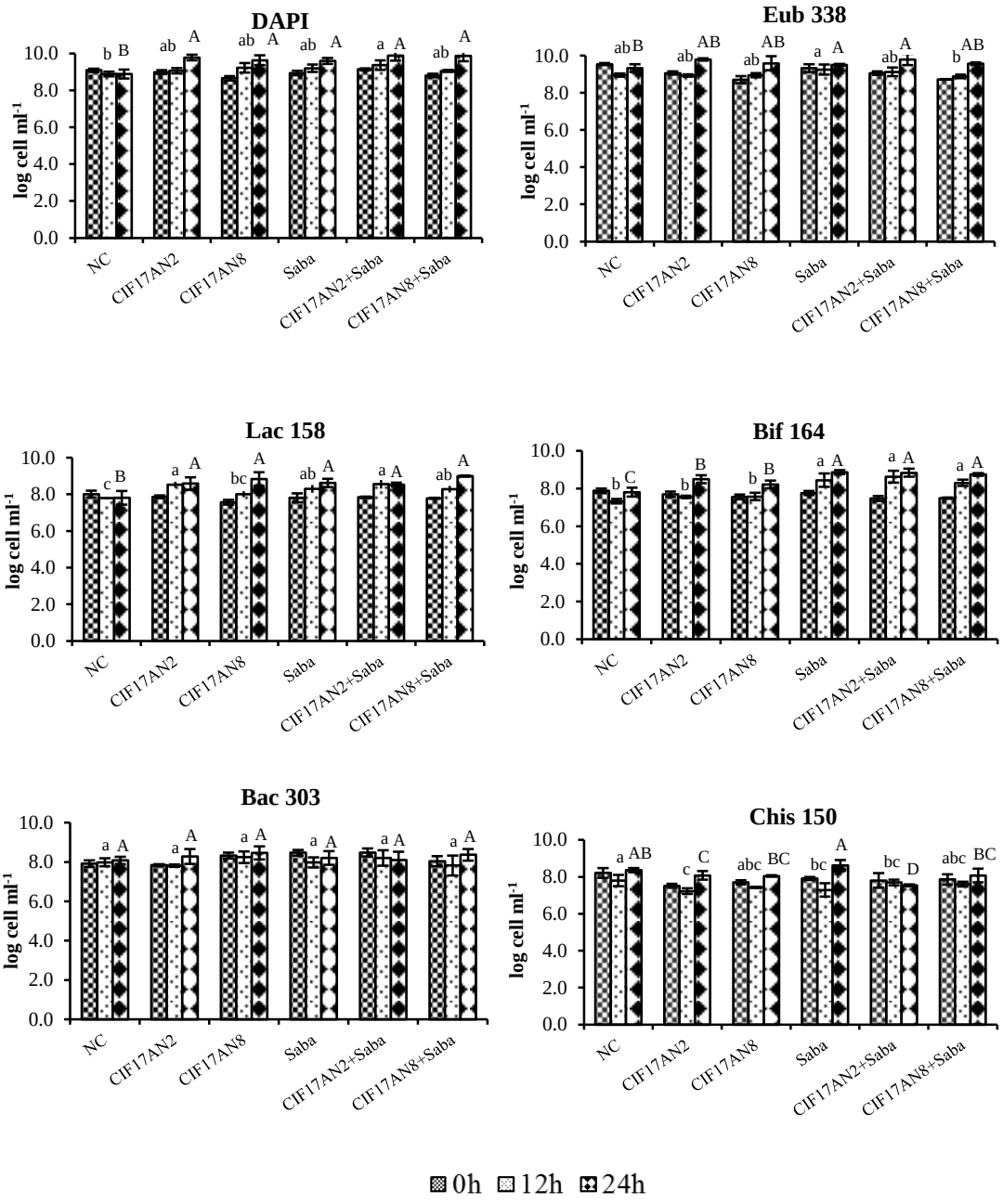


Figure 20. Effect of synbiotic combination of *L. plantarum* CIF17AN2 with saba extract, *L. plantarum* CIF17AN8 with saba extract and control probiotic and saba extract alone on total bacteria (DAPI), *Eubacterium* (Eub 338), *Lactobacillus/Enterococcus* spp. (Lac 158), *Bifidobacterium* (Bif 164), *Clostridium* (Chis 150) and *Bacteriodes* (Bac 303) in anaerobic mixed culture system.

5.5 Discussion

Prebiotics are nondigetible carbohydrate food component that are selectively fermented in the colon, increasing number of bifidobacteria and lactobacilli, which are believed to provides some degree of protection against pathogenic bacteria. As well, plant extracts in this study including saba extract, gros michel, mung bean, okra were able to support the growth of probiotic bacteria. Especially, saba and gros michel, the growth of probiotic bacteria in medium presenting of these prebiotic was more than 8 log CFU ml^{-1}. The moderate growth of probiotic bacteria was found in medium supplemented with mung bean, okra, and commercial prebiotic (GOS). In contrast, no or a bit of growth of probiotic bacteria was detected when inulin and FOS were used as carbon source. The growth of probiotic bacteria in different carbon sources was correlated with carbohydrate utilization and pH changing. The enormous carbohydrate consumption and dramatic decreasing in pH were found in saba and gros michel as carbon sources.

The data presented on growth levels, sugar consumption and changing pH indicated that probiotic bacteria were unable to metabolize inulin and FOS. High concentration of FOS and inulin still remined in medium. Only some strains were able to utilize FOS however, the level of consumption was very rare. The ability to use prebiotic as carbon source varied in probiotic strains. The lack of enzyme to hydrolyze the linkage resulted to low carbohydrate consumption for some probiotic. Chain length of the carbohydrate is also likely to be a contributory factor, since long chain oligosaccharides, with multiple branching, require more enzymatic hydrolysis by the organisms before its complete fermentation (Fook and Gibson 2002). However, the ability of probiotic bacteria to utilize FOS as carbon source has been reported (Pedreschi *et al.*, 2003). Likewise, *L. plantarum* 0407 combined with FOS showed the most effective at inhibiting *Escherichia coli*, *Clostridium jejuni* and *Salmonella enteritidis*. A significant, 6 log CFU ml^{-1} decrease in *E. coli* numbers was observed, whilst after the same time period, *C. jejuni* and *S. enteritidis* were undetectable (Fooks and Gibson, 2002).

The selectively stimulating the growth and/or activity of one of a limited number of bacteria in the colon and thus improve host health is one of prebiotic properties (Gibson, 1999). Due to the prebiotic effect of saba and gros michel to support the growth of probiotic bacteria resulted to antimicrobial activity of probiotic grown with these carbon

source. The preference growth, carbohydrate consumption in saba and gros michel resulted to low pH of supernatant that led to the inhibitory activity. Whereas, the absence of antimicrobial activity in some treatments could be due to low growth of bacterial cell, low carbohydrate utilization and no decreasing pH.

The antimicrobial activity in co-culture experiment was quite different from broth microdilution assay in 96 well plates. The 24 h cell free culture supernatant derived from cultivation of probiotic in minimal medium containing saba and gros michel showed inhibitory activity toward all three pathogenic bacteria while, in co-cultivation, only *L. platarum* CIF17AN2 and CIF17AN8 gave no growth of all pathogens within 24 h. However, the antimicrobial activity in micro-plate assay was only bacteriostatic. The antimicrobial activity of potential probiotic bacteria shown in 96 well plate assay and co-culture might be due to the production of antimicrobial compound like short chain fatty acid. Short chain fatty acids are the major fermentation product of carbohydrate metabolism and function as energy to the intestinal mucosa, stimulation of sodium and water absorption and lowering of the pH (Pan *et al*., 2009b). Therefore, bacteria grow poorly also result to low or no antimicrobial activity. The lowering of pH result to the ability to dissociate of organic acid (Skrivanova *et al*., 2006) Un-dissociated forms of organic acid penetrates the lipid membrane of the bacterial cell and dissociate within the cell. As bacteria maintain a neutral pH of the cytoplasm, the export of excess protons consumes cellular ATP and results in depletion of energy (Skrivanova *et al*., 2006). Another study has been shown that a low pH of the lactic acid producing bacteria culture condensate mixture able to inhibit *Salmonella enteritidis* (Park *et al*., 2005). Non-dissociated form of lactic acid triggers a lowering of the internal pH of the cell that causes a collapse in the electrochemical proton gradient in sensitive bacteria (Gonzalez *et al*., 2007).

From the result of co-culture study, we found that *L. plantarum* CIF17AN2 and *L. plantarum* CIF17AN8 in combination with saba extract showed effective synbiotic effect by inhibiting the growth of pathogenic bacteria. Therefore, these combinations were selected and studied for their efficiency on human gut micro flora in *in vitro* batch culture fermentation. In addition, the combination of saba extract and *L. plantarum* both strain CIF17AN2 and CIF17AN8 displayed potential synbiotic properties by significant increasing number of bifidobacteria which are claimed as beneficial bacteria. The beneficial intestinal flora protects the intestinal tract from proliferation or infection of harmful

bacteria (Mitsuoka, 1990). In addition, these combinations especially *L. plantarum* CIF17AN2 and saba extract were able to inhibit the growth of deleterious bacteria like clostridia which manifest pathogenicity when the host's resistance is decreased (Mitsuoka, 1990). The antimicrobial activity against enteropathogens also found in the combination of *L. plantarum* 0407 with oligofructose and *B. bifidum* Bb12 with a mixture of oligofructose and xylo-oligosaccharides (50:50 w/w) in batch fermenters (Fooks and Gibson, 2003). Unluckily, saba extract not only supported the growth of beneficial bacteria but undesirable bacteria (clostridia) also grew well in medium contained this extract. In contrast, Bialonska and colleague (2010) have reported, the commercial extract of pomegranate by-product (POMx) exposure enhanced the growth of only beneficial bacteria like total bacteria, *Bifidobacterium* spp. and *Lactobacillus* spp., without influencing the *Clostridium coccoides, Eubacterium recterium* rectal group and the *C. histolyticum* group.

From this result led to less interest in saba extract. However, there are many research have reported prebiotic substance from banana (Topping and Clifton, 2001; Topping *et al*., 2003; Fuentes-Zaragoza, *et al*., 2011). Due to, unripen banana contained starch which resist to human upper digestive system and was able to reach to the colon (Faisant *et al*., 1995). From these properties, let us interest in starch extracted from saba. Therefore, saba starch was extracted from unripens saba and combined with the potential probiotic *L. plantarum* CIF17AN2 and was used for further study.

5.6 Conclusion

Four plants extract including saba, gros michel, mung bean and okra exerted prebiotic characteristic by supporting the growth of probiotic bacteria. Among 4 of them, saba extract showed the best prebiotic properties. This prebiotic extract not only supported the growth of probiotic bacteria but also enhanced the antimicrobial activity of potential probiotic bacteria both in broth microdilution assay in 96-well plate and co-culture system especially, when it was combined with *L. plantarum* CIF17AN2 and *L. plantarum* CIF17AN8. Both combinations displayed potential synbiotic by rapid reducing *S. aureus* TISTR 1466, *E. coli* TISTR 780 and *Sal.* Typhimurium SA2093 level until undetected within 24 h. However, only the combination of saba extract and *L. plantarum* CIF17AN2 exerted properly synbiotic effect. This combination supported the growth of beneficial gut microflora and diminished the growth or had no positive effect on deleterious bacteria like

clostria and bacteroides. However, we found undesirable property of saba extract when it presented alone. This plant extract distinctly supported the growth of clostridia.

CHAPTER 6

PROTECTIVE EFFECT OF SABA STARCH ON SURVIVAL OF *LACTOBACILLUS PLANTARUM* CIF17AN2 DURING VACUUM DRYING AND STORAGE

6.1 Abstract

Banana starch was prepared from unripen saba (*Musa sapientum* (Linn)) (Kluai Hin). The protective effect of saba starch on survival of *Lactobacillus plantarum* CIF17AN2 (probiotic bacteria) during different drying processes (at 37 °C for 4 days; at 45 °C for 3 days; at the ambient temperature (~27 °C) for 5 days; vacuum drying in desiccator connected to suction pump for 4 days; and at 37 °C, 30 mm Hg in vacuum oven for 12 h), storaged at 4 °C and ambient temperature, and simulated human gastrointestinal transient was evaluated. The result showed that drying by using vacuum oven gave the highest survival of *L. plantarum* CIF17AN2 with survival rate 85.81% and 51.75% for the cell with and without saba starch addition, respectively ($P<0.05$). Whereas, the survival of *L. plantarum* CIF17AN2 from other drying methods was less than 65% and 50% in synbiotic product with and without of saba starch, respectively. Therefore, drying under vacuum oven was selected to develop synbiotic product. The result showed that saba starch adding synbiotic product significantly ($P<0.05$) increased the survival of *L. plantarum* CIF17AN2. Moreover, saba starch containing synbiotic product was able to prolong viability of probiotic bacteria during 8 weeks of storage by stabilizing its moisture content. Whereas, the viability of vacuum drying *L. plantarum* CIF17AN2 without saba starch addition was decreased due to the increasing of moisture content. Unfortunately, saba starch addition could not protect vacuum-dried probiotic bacteria from extreme condition in the upper part of human gastrointestinal tract. Nearly 50% reduction in cell viability was found in vacuum-dried synbiotic product after exposure to extreme conditions.

6.2 Introduction

Interests in the consumption of foods that contain probiotics are increasing and ranges of functional food that contain microorganisms have been developed (Ying *et al.*, 2010). Probiotic food products should contain at least 10^7 live bacteria per gram or per milliliter of product (Ying *et al.,* 2010). In addition, in order to produce the therapeutic benefits, suggested minimum level for probiotic bacteria in yoghurt is 10^5-10^6 viable cell ml^{-1} or gram of product (Dave and Shah, 1997). As well, the International Dairy Federation (IDF) suggests a minimum concentration of 10^7 CFU ml^{-1} until the date of the product expiration (Sultana *et al.*, 2000). Therefore, it is important that probiotic must be maintained their viability during manufacture, storage and delivery to the target site in the gastrointestinal tract to exert their health benefit. Preservation bacteria in frozen or drying form are relatively gave high levels of bacteria survival and maintenance of activity but, frozen cultures bear high storage and transportation costs in keeping cultures at very low temperatures such as -20 to -40 °C (Santivarangkna *et al*., 2006). Therefore, drying is another choice for preservation bacteria. However, a disadvantage of drying is the inactivation of bacteria during drying process. During thermal processing, stress caused by heat and by dehydration may be significant to the cells. Both forms of stress at lethal levels cause permanent loss of viability. Excessive heat causes denaturation of the structure of macromolecules or breaks the bonds between monomeric units. Stress caused by dehydration affects principally the cytoplasmic membrane by changing fluidity or the physical state of the membrane, in addition to causing lipid peroxidation. Use of additives such as carbohydrates and plant fibers might prevent membrane damage during freezing and dehydration processes (Linders *et al.*, 1997; Hongpattarakere *et al*., 2013). Wheat dextrin and polydextrose addition was proven to be the promising carriers during freeze-dried and storage to significantly improve viability of *Lactobacillus rhamnosus* (Saarela *et al*., 2006). Moreover, adhesion of *Lactobacillus casei* (LC-1) to oat bran and green banana starch was shown to protect this probiotic cells during vacuum drying, storage at different temperatures as well as exposure in simulated gastrointestinal condition (Guergoletto *et al.*, 2010).

Starch has a wide range of applications in the food and non-food products. The choice of starch in particular food product is governed by its physico-chemical

proportion (Suntharalingam and Ravindran, 1990). Banana though generally consumed as dessert fruit, also contains high content of starch when unripen and could be exploited as starch source. Its potential acceptance in food preparation is high due to absence of flavor. In addition, it is well established that variable amounts of starch in food can escape digestion in the human small intestine and pass into the colon; this fraction is referred to as resistant starch (RS) (Bello-Pérez *et al*., 1999). Unripened banana starch is very resistant to digestion in the rat and human small intestine (Bello-Pérez *et al*., 1999). In this study, the protective effect of saba starch on survival of *L. plantarum* CIF17AN2 during different drying processes, storage at refrigerated and unrefrigerated temperature, and sequential exposure to acid and bile salt conditions were investigated.

6.3 Materials and methods

6.3.1 Saba starch preparation

Banana starch was extracted according to a modification of a procedure of Aht-Ong and Charoenkongthum (2002). Unripen saba (*Musa sapientum* (Linn)) (Kluai Hin) was peeled and sliced into small pieces and then dried at 55 ºC for 7 h. The dried saba was soaked in 0.05 N NaOH for overnight. After that, the soaked saba was washed 3 times with water. The washed saba was mixed with water in ratio 1:1 and ground with blender until it was homogenized. The slurry was filtered through a sieve in pore size of 80, 120, and 170, respectively. The starch was washed several times with distilled water until the supernatant layer was substantially free of color. The starch solutions were left until precipitate and the water was drained. Starch was dried in an air oven at 65 °C. The dried starch was blended and separated with a sieve 230 pore size.

6.3.2 Probiotic bacteria

Probiotic *Lactobacillus plantarum* CIF17AN2 was isolated from infant feces and already screened for its probiotic properties. The probiotic *L. plantarum* CIF17AN2 was routinely cultured in MRS medium and incubated at 37 °C for 24 h.

6.3.3 Development of synbiotic product

The overnight culture of *L. plantarum* CIF17AN2 was centrifuged at 10,000 rpm. The bacterial cells were washed twice with phosphate buffer saline (PBS) pH 7.2 and then resuspended and adjusted to obtain 1.66×10^{10} CFU ml^{-1} (N_0) with the same buffer. Saba starch extracted from unripen saba was used as prebiotic fiber for protection of probiotic bacteria during drying process. Cell suspension of *L. plantarum* CIF17AN2 was added to saba starch in ratio 1:1 (v/w). After homogenization, the mixture was dried at different conditions; 1) at 37 °C for 4 days, 2) at 45 °C for 3 days, 3) at the ambient temperature (~27 °C) for 5 days, 4) vacuum drying in desiccator connected to suction pump (30 mmHg) for 4 days and 5) at 37 °C in vacuum oven (30 mmHg) for 12 h. Probiotic without saba starch addition was used as control. The synbiotic product was dried until a moisture content (AOAC,1999) of approximately 5.6% (Zayed and Roos, 2004). For cell recovery, the dried sample was brought to its original volume with PBS to obtain bacterial suspension, which was then serially diluted and transferred to the appropriate medium for viable counts on MRS agar (N_1). The results were expressed as % survival which was calculated from the following equation: % Survival = $(\log N_1/\log N_0) \times 100$

6.3.4 Shelf life of synbiotic product

The vacuum drying under vacuum oven gave the best cell survival so this drying method was chosen for development of synbiotic product. The cell suspension of probiotic was added to saba starch in ratio 1:1 (v/w) and dried under low pressure (30 mmHg) and moderate temperature (37 °C) for 12 h in vacuum oven. The dried sample was packed in aluminum foil-laminated polyethylene sachet bag and hermetically sealed. The product was kept at 4 °C and ambient temperature for 8 weeks. Every 7 days, a new package was opened and analyzed for cell viability by growing on MRS agar, moisture content according to AOAC (1999) method and water activity by using water activity meter (Novasina, Switzerland). The viability of probiotic before (N_0) and during storage periods (N_1) was counted. The percentage of survival was calculated according to the equation described in 6.3.3.

6.3.5 Sequential exposure to acid and bile salt of the synbiotic product

The vacuum-dried synbiotic product was firstly exposed to acidic condition (0.85% NaCl containing 3 g l^{-1} pepsin (Sigma, Geramany) and adjusted to pH 2 with HCl) and bile salt (0.3% ox-gall bile and 3 mg ml^{-1} pancreatin (Sigma, USA)). The test tubes were incubated at 37 °C for 3 h and 6 h with gentle agitation (by shaker) for acid and bile conditions, respectively. Thereafter, the pretreated cell was then serially diluted and transferred to the appropriate medium for viable counts on MRS agar. Overnight culture of *L. plantarum* CIF17AN2 was used as control. The viability of probiotic bacteria before (N_0) and after (N_1) exposure to simulated human gastrointestinal conditions was counted. The percentage of survival was calculated according to the equation described in 6.3.3.

6.4 Results

6.4.1 Determination the survival of probiotic bacteria after drying

Survival of *L. plantarum* CIF17AN2 after drying was determined by counting on MRS agar and the result was shown in Figure 21. *Lactobacillus plantarum* CIF17AN2 was able to survive in all drying method except the one dried at 45 °C. Vacuum drying under vacuum oven gave significantly the survival of *L. plantarum* CIF17AN2. The survivability of probiotic bacteria from this drying method was 85.81% and 51.75% for treatment with and without saba starch addition, respectively. Drying of *L. plantarum* CIF17AN2 with saba starch addition at 37 °C for 4 days, at the ambient temperature (~27 °C) for 5 days and vacuum drying in desiccator connected to suction pump for 4 days also able to provide cell survivability. The survival of *L. plantarum* CIF17AN2 with these drying methods was 55.45, 66.76 and 63.99%, respectively. While, drying of *L. plantarum* CIF17AN2 with these drying methods provided the cell viability only 20.30, 44.40 and 37.75%, respectively. This indicated that saba starch addition was significant increase the survival of probiotic bacteria in all drying methods.

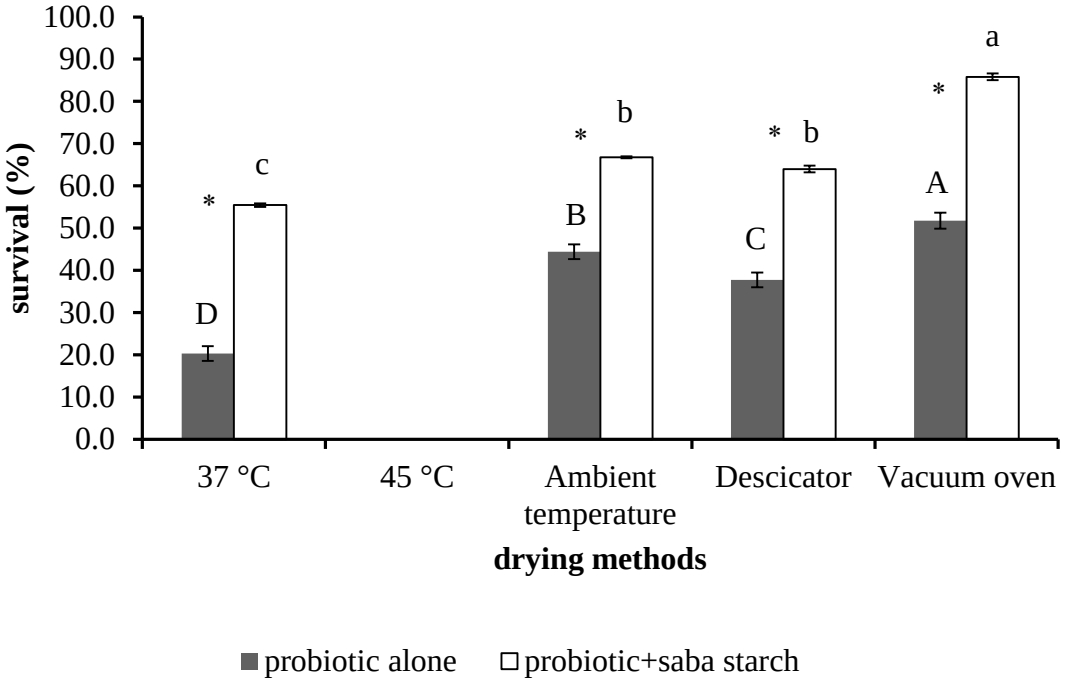


Figure 21. Survival percentage of *L. plantarum* CIF17AN2 after drying at 37 °C for 4 days; 45 °C for 3 days; the ambient temperature (~ 27 °C) for 5 days; desiccator connected to suction pump for 4 days; and at 37 °C in vacuum oven for 12 h both in the presence and absence of saba starch. Different letters within the same bar color indicate significant difference ($P<0.05$). The asterisk (*) indicates the significant difference between control and the saba starch addition.

6.4.2 Shelf life of synbiotic product

The hermetically sealed in aluminum foil bag vacuum drying synbiotic product was evaluated for its biological and physiological characteristic during 8 weeks storage at 4 °C and ambient temperature. There was no decrease in cell viability of synbiotic product and the control treatment (without saba starch) storage at 4 °C and ambient temperature at the first three weeks (Figure 22). However, the sharply decreased in viable cell was detected after 3 weeks storage in the control treatment stored at both temperatures. In contrast, *L. plantarum* CIF17AN2 was maintained its viable number at the same level as the beginning of storage until 6 weeks in saba starch adding synbiotic product stored at 4 °C. At the end of storage, the log reduction of *L. plantarum* CIF17AN2 in saba

starch adding synbiotic product was only 0.5 log CFU ml^{-1} and 1 log CFU ml^{-1} of storage temperature at 4 °C and ambient temperature (~27 °C), respectively.

Moisture content of synbiotic product was quite stable at both storage temperatures during 8 weeks (Figure 23A). Whereas, obvious increase in moisture content was detected in vacuum-dried *L. plantarum* CIF17AN2 without saba starch addition and stored at non-refrigerated temperature. As well, there was no increase in water activity of synbiotic product stored at both temperatures during 7 weeks of storage period (Figure 23B). Whereas, a bit increase in water activity of synbiotic product was found at the end of storage. Due to the limit of water activity machine therefore we could not measure low amount of vacuum-dried probiotic bacteria (without saba starch addition).

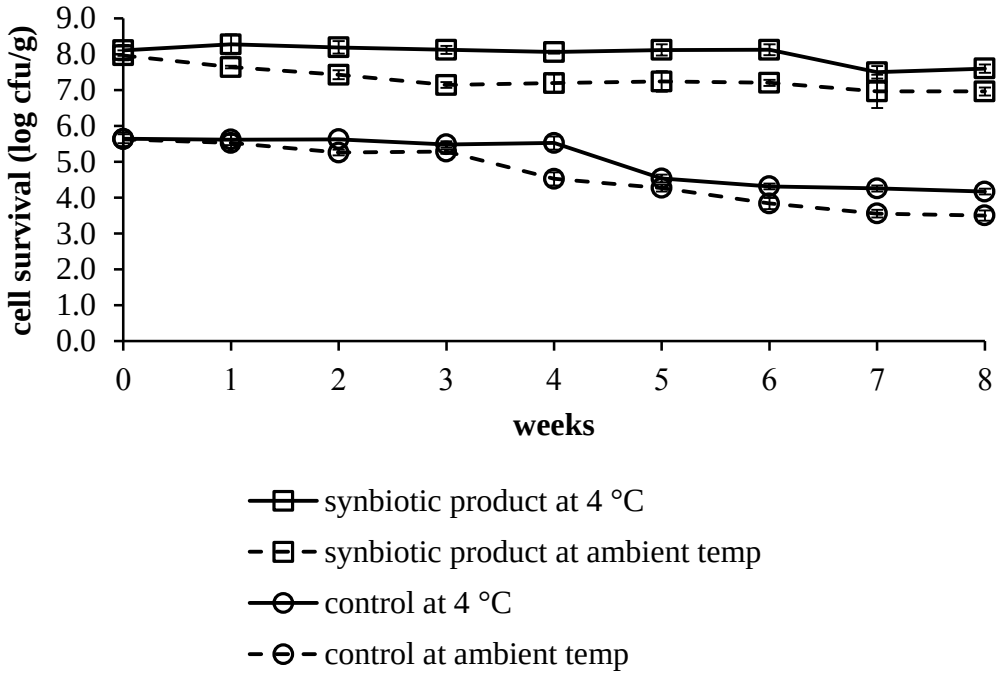


Figure 22. Shelf life of vacuum-dried synbiotic product (*L. plantarum* CIF17AN2 and saba starch) and control (vacuum-dried *L. plantarum* CIF17AN2 without saba starch adding) during storage at 4 °C and ambient temperature (~27 °C) for 8 weeks.

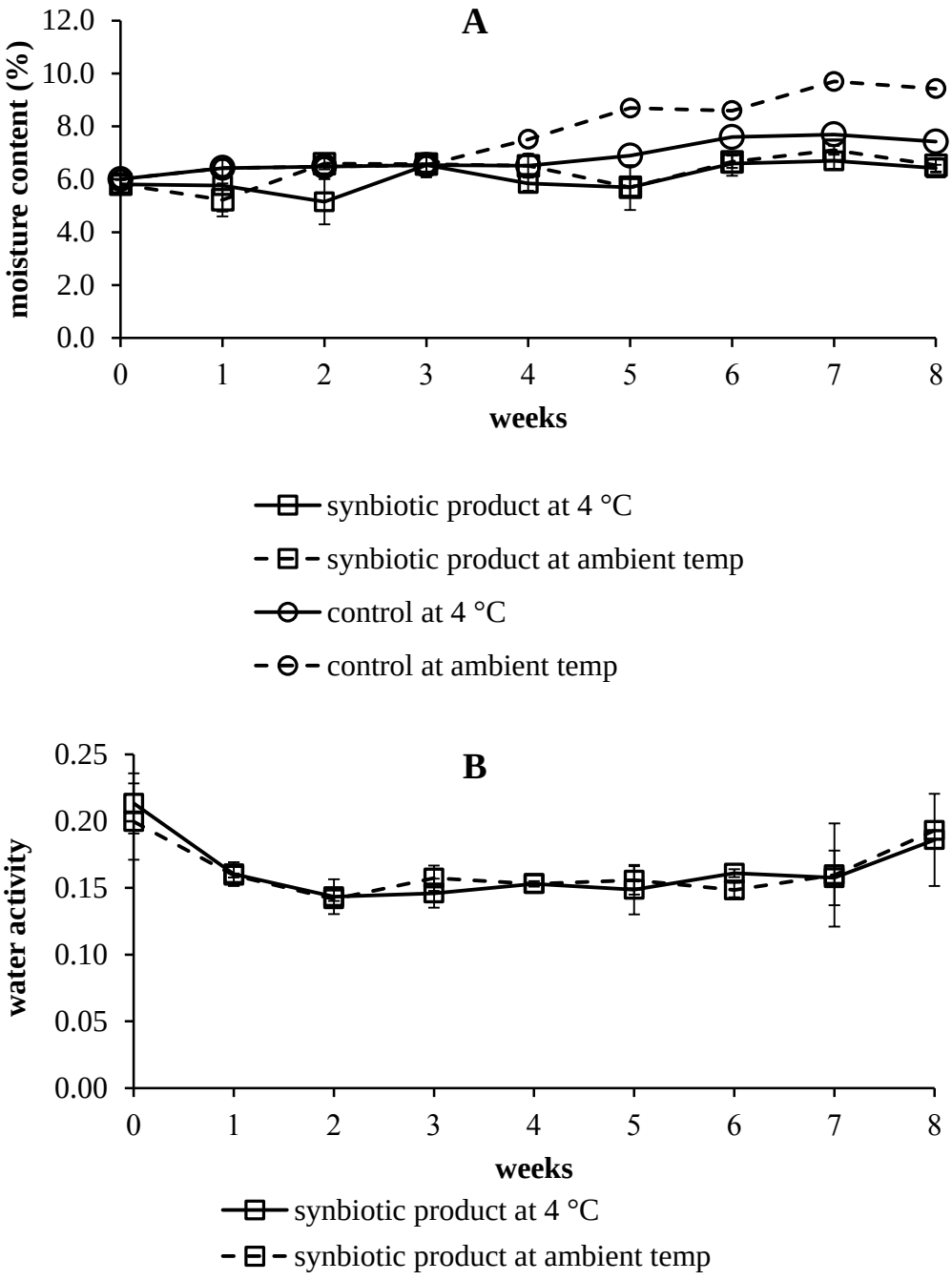


Figure 23. Moisture content (A) and water acitivity (B) of vacuum-dried synbiotic product (*L. plantarum* CIF17AN2 and saba starch) and control (vacuum-dried *L. plantarum* CIF17AN2 without saba starch adding) during storage at 4 °C and ambient temperature (~27 °C) for 8 weeks.

6.4.3 Survival of vacuum-dried synbiotic product after exposure to condition simulated upper part of human gastrointestinal tract

Survival of *L. plantarum* CIF17AN2 in vacuum-dried synbiotic product after exposure to acid (pH 2 + 3 mg ml^{-1} pepsin) for 3 h and followed by bile salt (0.3% ox-gall bile + 3 mg ml^{-1} pancreatine) for 6 h was determined. Vacuum-dried *L. plantarum* CIF17AN2 alone and normal cell (overnight culture of *L. plantarum* CIF17AN2) were used as control. *Lactobacillus plantarum* CIF17AN2 in vacuum-dried synbiotic product was sensitive to these conditions (Figure 24). The viability of *L. plantarum* CIF17AN2 was significantly decreased. The reduction of vacuum-dried *L. plantarum* CIF17AN2 in synbiotic product after exposure to gastric condition followed by bile salt condition was 3.17 and 3.47 log CFU ml^{-1}, respectively. As well, vacuum-dried *L. plantarum* CIF17AN2 alone was also sensitive to this transit condition. On contrast, the *L. plantarum* CIF17AN2 in control treatment was able to withstand in gastric fluid and gastric fluid followed by bile salt fluid with the reduction only 0.87 and 2.03 log CFU ml^{-1}, respectively.

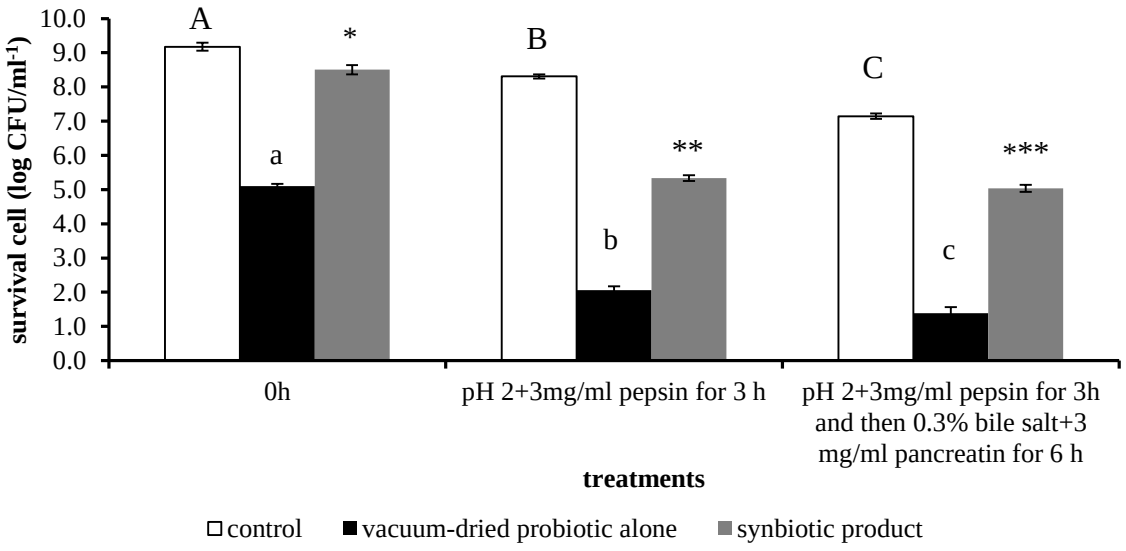


Figure 24. Survival of the vacuum-dried synbiotic product (*L. plantarum* CIF17AN2+saba starch) and vacuum-dried *L. plantarum* CIF17AN2 alone after sequential exposure to simulated gastric fluid for 3 h followed by bile salt fluid for 6 h. Cell pellet of overnight culture of *L. plantarum* was used as control. Different letters and sign numbers within the same color bar indicate significant difference ($P<0.05$).

6.5 Discussion

Most commercial starter cultures are preserved in frozen or freeze dried form because of relative high levels of survival and maintenance of activity. Frozen cultures bear high storage and transportation costs by keeping cultures at very low temperatures such as -20 to -40 °C (Santivarangkna *et al*., 2006). Freeze drying has high production costs because of the energy requirements. In this study, we tried to find alternative drying methods with lower cost. We found that the vacuum dehydration under low pressure with room temperature gave the highest cell survivability, while the other methods 45 °C for 3 days, 37 °C for 5 days, room temperature for 5 days, and suction drying for 5 days gave no or low viability of probiotic bacteria. Vacuum-dried is one of the promising processes, which the moisture evaporation rate is higher and conditions of low pressure allow moderate temperature to be used where the thermal inactivation of cell can be avoided (Santivarangkna *et al.,* 2006; Guergoletto *et al*., 2010). The high temperature used such 45 °C for 3 days maybe caused to denature of critical cell component mainly DNA and RNA (Linders *et al*., 1997). However, a much loss in cell viability of *L. plantarum* CFI17AN2 after vacuum drying was found when it presented alone. The removal of water profoundly from bacterial cell alters the physical properties of membrane phospholipids, leading to destructive events such as fusion, liquid crystalline to gel phase transitions, and elevation of permeability. During rehydration, the dry membrane retunes from gel phase to liquid crystalline phase, and in course of this process, there are membrane leakages from the packing defects (Crowe *et al*., 1987). In addition, survival of microorganisms during drying processes and subsequent storage depends on factors, such as species and strain, drying condition, inoculums and culture media (Otero *et al*., 2007; Hongpattarakere *et al*., 2013).

Therefore, to improve survival of probiotic bacteria, saba starch was used as protective prebiotic fiber during vacuum-dried. The inclusion of saba starch as a protective substance was effective to improve the survival of probiotic bacteria during vacuum-dried. The green banana starch contributing the greater survival of *Lactobacillus casei* (LC-1) during drying under vacuum over at 45 °C, 0.09 MPa for 25 h has been reported (Guergoletto *et al*., 2010). However, the survivability of our vacuum-dried probiotic bacteria with saba starch addition (85.81%) was higher than report of Guergoletto and colleague (2010) (76%) even if, the experiment was set to allow adhesion of *L. casei* (LC-

1) to green banana flour at 25 °C for 1 h. Vacuum-dried *L. plantarum* CIF17AN2 alone lost its viability during 8 weeks storage at both refrigerated and non-refrigerated temperatures. This may be due to increase of moisture content. The mechanism behind the loss of cell viability during the storage period depends on series of factors, including temperature, moisture content, protectors and oxidative stress (Fu and Chen, 2011). Increase of moisture content results to loss of *Lactobacillus* viability during storage (Kurtmann *et al*., 2009). The loss of probiotic viability during storage can be solved by adding of saba starch. Saba starch added in synbiotic product resulted to only a bit increase in moisture content during storage for 8 weeks at refrigerate and room temperature. In addition, water activity of this product was less than 0.2. Dry product with low water activity (a_w <0.25) is generally expected shelf-life of months. Cell survival is particularly affected when the food matrix has an elevated water activity (a_w > 0.25) (Weinbreck *et al.*, 2010).

However, saba starch could not protect vacuum-dried *L. plantarum* CIF17AN2 from simulated extreme condition in upper part human gastrointestinal tract. This may be due to some part of important structure of this probiotic bacteria was damaged during drying process. Although the exact mechanisms of cell inactivation during drying processes are not yet fully elucidated, it is known that bacterial cells consist of 70% to 95% water, and its removal poses serious physiological obstacles to the survival of cells (Santivarangka *et al.*, 2008).

The cytoplasmic membrane is generally considered to be the main site of dehydration inactivation (Gardiner *et al*., 2000; Santivarangka *et al.,* 2006). Stress caused by dehydration affects principally the cytoplasmic membrane by changing fluidity or the physical state of the membrane, in addition causing lipid peroxidation. The damage of phospholipid bilayers at cell cytoplasmic membrane from dehydration was due to the removal of hydrogen-bonded water from the phospholipid bilayer which results to increase in the head-group packing of membrane lipid, leading to increase opportunities for van der Waals interaction among hydrocarbon chains. Therefore, the lipids change from liquid crystalline to gel phase in dry membranes because of the increase in melting temperature (Tm) of membrane lipid. During rehydration, the dry membrane returns from gel phase to liquid crystalline phase, and in course of this process, there are membrane leakages from the packing defects (Santivarangkna *et al.,* 2006). Besides the cell membrane, the cell wall and also cellular protein can possibility be a target for the dehydration inactivation

(Santivarangkna *et al.*, 2006). Therefore, leakage of cell increased in the sensitivity of cells to extreme condition. This result is in agreement with Santivarangkna and colleague (2006) who reported that the damaged cell cytoplasmic membrane increased the sensitivity to chemicals such as NaCl, Oxgall and lysozyme. In contrast with our result, vacuum-dried *Lactobacillus casei* (LC-1) adhered to oat bran had greater viability (7.1 log CFU g^{-1}) than in the free cell (2.4 log CFU g^{-1}). Allowing the adhesion of *L. casei* (LC-1) to oat bran fiber at 25 °C for 1 h was effective in protecting of this probiotic strain during vacuum-dried process as well as in simulated gastrointestinal conditions. Scanning electron microscopy observed no morphological changes of *L. casei* (LC-1) adhesion to this fiber after drying (Guergoletto *et al*., 2010).

6.6 Conclusion

This study demonstrated that among different drying methods, drying under vacuum oven gave the highest probiotic survivability. Saba starch was a perfect fiber for protection *L. plantarum* CIF17AN2 during vacuum-dried process. In addition, it also helped probiotic bacteria maintaining its viability during 8 weeks storage at refrigerated and unrefrigerated temperature. However, it could not be able to protect dried synbiotic product from low pH and bile salt conditions. Therefore to improve stability of synbiotic product, the addition of supporters, allowing of probiotic bacteria adhere to prebiotic fiber or pre-adapted of probiotic bacteria to the stress inherent before drying process will be further choices for development of our synbiotic product.

CHAPTER 7

SYNBIOTIC EFFECT OF *LACTOBACILLUS PLANTARUM* CIF17AN2 AND BANANA STARCH ON DOMINANT GUT MICROFLORA AND GROWTH OF *SALMONELLA ENTERICA* SUBSP. *ENTERICAL* SEROVA TYPHIMURIUM SA2093 IN CHALLENGED SYSTEM UNDER CONDITION SIMULATED HUMAN PROXIMAL COLON

7.1 Abstract

Saba starch (*Musa sapientum* (Linn)) (Kluai Hin) both in combination with and without *Lactobacillus plantarum* CIF17AN2 was studied for its efficiency on dominant human gut microflora and also the growth of *Salmonella enterica* subsp. *enterica* serovar Typhimurium SA2903 in challenged system under condition simulated human gastrointestinal tract both with un-controlled and controlled-pH. Fluorescent *in situ* hybridization (FISH) exposed prebiotic effect of saba starch and synbiotic effect of developed synbiotic combination of saba starch and *L. plantarum* CIF17AN2 which selectively supported the growth of beneficial bacteria like lactobacilli and bifidobacteria Saba starch did not enhance the growth of undesirable bacteria like bacteriodes and clostridia in both systems. These beneficial effects resulted to lowering pH of culture medium and production of antimicrobial substances especially short chain fatty acid to inhibit the growth of *Sal*. Typhimurium SA2093. Denaturing gradient gel electrophoresis (DGGE) with nested PCR approach revealed noticeably modification of bifidobacteria population in saba starch and synbiotic supplemented system whereas, lactobacilli profile was not different between supplemented and control group.

7.2 Introduction

Starch is the major dietary component in all human population (Bello-Pérez *et al*., 1999). Starch is deposited in fruit in the form of granules, partially crystalline. Its morphology, chemical composition and supermolecular structure are characteristic of each particular plant species. Starch is classified based on its functionality into two major high molecular weight carbohydrate components: amylose and amylopectin. These macromolecules are physically organized into the granular structure (Bello-Pérez *et al.,* 1999). Starch is thought to be completely digested in the human small intestine because very little starch is found normally in feces. However, a substantial data studied *in vitro* and *in vivo* both in animal and intact human volunteers as well as those with defunctioning bowel surgery (ileostomates) has shown that a substantial proportion of the starch escapes into the large bowel (Topping and Clifton, 2001; Topping *et al*., 2003). This starch is termed resistant starch (RS) which includes the portion of starch that can resist digestion by human pancreatic amylase in the small intestine and thus, reach the colon (Faisant *et al*., 1995; Bello-Pérez *et al.,* 1999; Fuentes-Zaragoza *et al*., 2011). Feeding of rice porridge with high content of resistant starch appears to modify the autochthonous porcine large-bowel microflora favorably through lowering *Escherichia coli* and coliform numbers (Topping *et al*., 2003). Resistant starch occurs basically in all starchy foods but not in a fixed quantity (Fuentes-Zaragoza *et al*., 2011). Surprisingly, banana starch has been reported that contained slowly digestible carbohydrates and starch component resistant to digestion of amylases (Faisant *et al*., 1995; Juarez-Garcia *et al*., 2006). Unripen banana, constitutes 40-50% resistant starch, while simple carbohydrates (glucose, fructose, sucrose, etc.) are very scarce (Juarez-Garcia *et al.,* 2006). The resistant starch showed a great potential as prebiotic and colonic food, which is emerging as a major factor in the bacterial ecology of the human hindgut. Interest in resistance starch grew from the fermentation of complex carbohydrate by large bowel microflora. The metabolic end product of starch fermentation, especially short chain fatty acid (SCFA) have emerged as important metabolic for colonocytes as well as having specific actions that promote normal colonic function. Besides, prebiotic, probiotics and the combination of both namely synbiotic are known to have a role in prevention or treatment and relief of gastrointestinal disorder (Guarner and Malagelada, 2003). In addition, consumption of these beneficial substances

also improves host health by promoting the growth of exogenous bacteria (mostly species of lactic acid bacteria) contrasted with the putrefactive action of the resident flora (Topping *et al.,* 2003). Consumption of synbiotic containing *Bifidobacterium bifidum, Bifidobacterium lactic*, and oligofructose in elderly persons increased the size and diversity of protective fecal bifidobacterial population, which are often very much reduced in older people (Bartosch *et al.,* 2005).

Salmonella is an enteric pathogen that colonizes the intestinal tract of a variety of animals, especially humans and poultry and also cause of millions of case of gastroenteritis and food-borne illness each year (Palmer *et al.,* 2000; Deng *et al.,* 2007). There are some evidences indicating that large intestine is involvement in human *Salmonella* Typhimurium pathogenesis. Biopsies taken from patients indicate that the large intestine (i.e. colon) is likely the primary site of involvement during severe and/or fatal enteric infections with this pathogen (zu Bentrup *et al*., 2006). The large intestine can be divided into 5 parts, the ascending, transverse, descending and sigmoid colons and the rectum. The proximal colon (right side) have plenty of available dietary nutrients therefore, the microorganism resident in this region grow at a fast rate, causing a decreasing in pH (5-6) as result of intense short chain fatty acid production (Guarner and Malagelada, 2003).

Although, rarely work study the effect of prebiotic/probiotic or the synbiotic on diarrhea pathogenic bacteria. However, inhibitory effect of probiotic, prebiotic or synbiotic on pathogenic bacteria have also been reported (Liévin *et al.,* 2000; Gill *et al.,* 2001; Sazawal *et al*., 2006; Revolledo *et al*., 2009; Grandy *et al*., 2010; Honda *et al.,* 2011). Therefore, in this study we investigated the effect of saba starch alone and in combination with probiotic *L. plantarum* CIF17AN2 on the dominant human gut microflora and growth of S*almonella enterica* subsp. *enterica* serovar Typhimurium SA 2093 in challenging system under simulated proximal region of human gastrointestinal tract.

7.3 Materials and Methods

7.3.1 Microorganisms

Lactobacillus plantarum CIF17AN2 was isolated from healthy infant feces and routinely cultured in MRS broth (Himedia, Mumbia India) and incubated at 37 °C for 24 h. *Salmonella enterica* subsp. *enterica* serovar Typhimurium SA2093 was derived from

the Microbiological Laboratory of Songklanakarind Hospital, Songkhla, Thailand. The pathogen was cultured in Muller Hinton broth (MHB; Himedia, Mumbai, India) and incubated at 37 °C for 24 h.

7.3.2 Saba starch preparation

Banana starch was extracted according to a modification of a procedure of Aht-Ong and Charoenkongthum (2002). Unripen saba (*Musa sapientum* (Linn)) (Kluai Hin) was peeled and sliced into small pieces and then dried at 55 ºC for 7 h. The dried saba was soaked in 0.05 N NaOH for overnight. After that, the soaked saba was washed 3 times with water. The washed saba was mixed with water in ratio 1:1 and ground with blender until it was homogenized. The slurry was filtered through a sieve in series pore size of 80, 120, and 170 pore size. The starch was washed several times with distilled water until the supernatant layer was substantially free of color. The starch solutions were left until precipitate and the water was drained. Starch was dried in an air oven at 65 °C. The dried starch was blended and separated with amesh size 230. The saba extract was obtained from ethanol (50% v/v) extraction of ripe saba. The crude extracts were purified by precipitating twice in cool ethanol (final concentration of 80%). The purified extracts were freeze dried to obtained dry powder (Wicheinchot *et al.*, 2011).

7.3.3 Fecal slurry preparation

Fecal slurry (10% (w/v)) was prepared using fresh stool samples from 3 healthy donors (who had not taken antibiotics for 3 months prior to start the study). The fecal samples were mixed together and diluted with 0.1 mol l^{-1} phosphate buffer pH 7.2 (Oxoid Limited, England) containing 0.05% L-cysteine (Sigma, Japan) then was mixed in a stomacher for 2 min. The slurry was poured into the sterile container for use as an inoculum.

7.3.4 Development of synbiotic product

Overnight culture of *L. plantarum* CIF17AN2 was centrifuged and washed twice with PBS pH 7 and then resuspended in the same buffer to adjust the concentration of 10^{11} CFU ml^{-1}. The probiotic suspension was added to saba starch in ratio 1:1 (v/w). After

homogenization, the samples were dried in a vacuum oven at 37 °C, 30 mm Hg (Napco) for 12 h (moisture content of approximately 5.6 %) (Zayed and Roos, 2004).

7.3.5 Effect of probiotic, prebiotic and synbiotic supplement on the survival of *Sal. enterica* subsp. *enterica* serovar Typhimurium SA2093 and dominant fecal microflora in the challenged system

Salmonella Typhimurium SA2093 was challenged in the 6 treatments of basal medium consisting of 1) mixed fecal sample; 2) mixed fecal sample with addition of *L. plantarum* CIF17AN2; 3) mixed fecal sample with saba extract; 4) mixed fecal sample with saba starch; 5) mixed fecal sample with probiotic and saba extract; 6) mixed fecal sample with probiotic and saba starch. The negative control (NC) treatment was the minimal medium consisting of mixed fecal sample alone. Meanwhile the effect on the dominant human gut bacteria in the mixture of fecal microflora with the *Salmonella* challenge was also investigated. The system was operated in 50 ml serum vial. The vials were filled with basal growth medium then flushed with O_2-free N_2 to provide anaerobic condition and sealed with rubber stopper and secured by aluminum cap. The medium contained, per liter, 2 g of peptone water, 2 g of yeast extract, 0.1 g of NaCl, 0.04 g of K_2HPO_4, 0.01 g of $MgSO_4 \cdot 7H_2O$, 0.01 g of $CaCl_2 \cdot 6H_2O$, 2 g of $NaHCO_3$, 0.005 g of hemin, 0.0 5 g of L-cysteine hydrochloride, 0.5 g of bile salts, 2 ml of tween 80, 10 μl of vitamin K and 4 ml of 0.025% (w/v) resazurin solution. The five milliliter of fecal slurry was injected into each serum vial. Stock carbohydrate (10%) was prepared by dissolving saba extract and saba starch in sterile basal medium and then 5 ml was added into each vial to give final concentration of 1% (based on total sugar content). Overnight cultures of *L. plantarum* CIF17AN2 and *Sal.* Typhimurium SA 2093 was centrifuged and resuspended in sterile basal medium and then 1 ml of each was added into injection vial to give final concentration of 1×10^7 CFU ml^{-1}. All treatments were made at triplicate. The vial was incubated at 37 °C and samples were taken at 0, 6, 12, 24, 36 and 48 h for determination of dominant bacteria by FISH technique, growth of *Sal.* Typhimurium SA 2093 by culturing on Xylose lysine deoxycholate (XLD) and pH changing.

7.3.5.1 Quantification of dominant fecal microflora by FISH technique

Samples (375 µl) were added to 1.125 ml of 4% (w/v) filtered paraformaldehyde (chilled), mixed and stored at 4°C overnight to fix the cell. The fixed cells were then centrifuged at 12,000 rpm (Eppendorf, Germany) for 5 minutes and washed twice with cool filtered sterilize 0.1 mol l^{-1} PBS, pH 7.2 and resuspended with 150 µl PBS. After that, ethanol (150 µl) was added then mixed thoroughly. The fixed samples were stored at -20°C at least 1 h or until further analysis, which is no longer than 3 months. The fixed cells were diluted to obtain appropriate dilution. The 20 µl of suitable dilutions were placed onto the well of Teflon- and Poly-L-lysine coated slides. The slides were placed on slide dryer (46-50 °C, 15 min) followed by dehydration in alcohol series (50, 80 and 90% ethanol) for 3 min of each concentration and finally dried on slide dryer. Lysozyme treatment was required for *Lactobacillus*/*Enterococcus* before dehydration. Pre-warmed hybridization buffer was mixed with 5 µl of genus-specific 16S rRNA-target oligonucleotide probes (50 ng μl^{-1}). Fluorescent dye Cy3 labeled oligonucleotide probe specific for the different bacterial groups were Bif 164 (5'-CATCCGGCAT TACCACCC-3′) Bac 303 (5′-CCAATGTGGGGGACCTT-3′), Lab 158 (5′-GGTATTAG CAYCT TCCA-3′), chis 150 (5′ TTATGCGGTATTAATAT (C/T)CCTTT-3′), Eub 338 (5′-GCTGCCTCCCGTAGGAGT-3′) specific for *Bifidobacterium, Bacteroides, Lactobacillus/Enterococcus* spp., *Clostridium histolyticum* group, and *Eubacterium* group, respectively (Rycroft *et al*., 2001; Rochet *et al*., 2004; Al-Tamimi *et al*., 2006; Mandalari *et al*., 2007) and nucleotide target 4′,6-diamidino-2-phenylindole (DAPI) (Sigma, Sigapore) dye was used for total bacterial counts. The probe solutions (50 µl) were added onto each well, and allowed to hybridize for 4 h at 46 °C for Eub 338 and Bac 303 probes; 50 °C for Lac 158, Bif 164 and Chis 150 probes. After hybridization, the slides were washed with pre-warmed washing buffer, (0.9 mmol l^{-1} NaCl, 20 mmol l^{-1} Tris-HCl, pH 7.2) containing 20 µl DAPI solution (500 ng μl^{-1}) for 15 min at 48 or 50°C depending on the probe. The slides were dipped into cold distilled water and quickly dried. Five microliters of antifade (Fluka, Singapore) were then added onto slide well and covered by cover slide before visualization with fluorescence microscope (Nikon Eclipse 80i, USA). The DAPI stained

cells were examined under UV light and hybridized cells viewed using a Cy3 filter. A minimum of 15 fields, each containing 10-100 cells, was counted for each well.

7.3.5.2 Determination growth of *Sal*. Typhimurium SA2093

The samples were serially diluted to obtain appropriate dilution. XLD medium was then inoculated with 100 µl of diluted samples. The medium was spread until dry. Plates were incubated at 37 °C for 18-24 h. After incubation, presumptive colony of *Sal.* Typhimurium SA2093 (pink colony with black at the center) was counted.

7.3.6 Effect of the synbiotic supplement on growth of *Sal. enterica* subsp. *enterica* serovar Typhimurium SA2093 and dominant fecal microflora in the challenged system under simulated human proximal colon condition (pH control).

Effect of developed synbiotic (combination of saba starch and *L. plantarum* CIF17AN2) on dominant human gut microflora and growth of *Sal.* Typhimurium SA2093 was operated in challenged system under simulated proximal colon of human gastrointestinal tract with pH controlled at 5.5. *Salmonella* Typhimurium SA2093 was challenged in the 3 treatments of basal growth medium consisting of 1) mixed fecal sample; 2) mixed fecal sample with addition of saba starch; 3) mixed fecal sample with addition of developed synbiotic product. Water-jacketed fermenters were filled with 135 ml of sterilize basal medium. Fecal slurry (10%) was added into each vessel, saba starch was added to give final concentration 1%. Probiotic bacteria and pathogen were added to give final concentration of 10^7 CFU ml^{-1}. All vessels were magnetically stirred and the temperature was controlled at 37 °C by a circulating water bath. Culture pH was automatically controlled at 5.5 and anaerobic condition was maintained by sparking the vessels with oxygen-free nitrogen gas at 15 ml min^{-1}. Samples (5ml) were taken from each vessel at times 0, 3, 6, 9, 12, 18, 24 and 48 h for enumeration of dominant fecal microflora by FISH technique as method described in 7.3.5.1, investigation survival of *Sal.* Typhimurium SA2093 on XLD according to method 7.3.5.2, SCFA production by Gas chromato graphy (GC) and the changing of lactic acid bacteria and bifidobacteria profile by using DGGE.

7.3.6.1 Short chain fatty acid analysis

Sample (1ml) from each fermentation fluid was centrifuged at 12,000 rpm for 15 min to remove bacterial cell and other particulate materials. The obtained clear

supernatant was then filtered through a 0.45 µm syringe filter (Sartorius stedium Biotech). Short chain fatty acids including acetic, propionic, butyric and lactic acids were estimated by Gas Chromatography (gilent technology 7890A Network GC System, USA) with flame ionization detector (GC-FID). The GC separations were done using HP-INOWEX poly ethylene glycol (HP 19091N-133E) with 30 m × 250 µm × 0.25 µm. Helium was used as carrier gas with total flow at 104.5 ml min^{-1} and septum purge flow at 3 ml min^{-1}. The optimum temperature regime for the best separation of acids was 80 °C for 1 min then 20 °C min^{-1} to 120 °C for 1 min then 7 °C min^{-1} to 170 °C. The head pressure was set at 8.5 psi with splitless injection mode. The injection temperature and the detector were maintained at 260 °C. Glacial acetic acid AR grad (Lab-scan, Ireland), butyric acid (Panreac, E.U), propionic acid (Fluka, Germany) and L-(+)-lactic acid (Fluka, Germany) were used as standards. The GC condition was followed from Sheveleva and Ramenskaya (2001).

7.3.6.2 PCR-DGGE analysis

To investigate LAB and bifidobacteria profile in system simulated human proximal colon and supplemented with developed synbiotic (*L. plantarum* CIF17AN2 and saba starch) or prebiotic (saba starch), the microbial genomic DNA was extracted. The nested PCR was performed to amplify target DNA. The first round PCR to amplify the 16S rDNA of LAB was performed with 27-f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492-r (5'-GGTTACCTTGTTACGACTT-3') primers (Zhang *et al.*, 2007) while the 16S rDNA of bifidobacteria was amplified by primers Im26-f (5′GATTCTGGCTCAGGATGAACG-3′) and Im3-r (5′-CGGGTGCTICCCCACTTTCATG-3′) (Satokari *et al*., 2001). PCR volumes of 25 µl contained 12.5 µl red dye PCR master mix (Merck), 2.5 µl of each primer (2 mM), 0.5 µl DNA templates and 7 µl sterile Milli-Q water. The first round PCR products of the 16S rDNA were used as templates in the next amplification. The variable V3 region (380 bp) of 16S rDNA of LAB was amplified with Lac1 forward (5′-AGCAGTAGGGAATCTTCCA-3') and Lac2 reward (GC-clamp-5′-ATTYCACCGCTA CACATG-3′) (Walter *et al*., 2001; Zhang *et al*., 2007; Minervini *et al*., 2010), whereas Bif164-f (5′-GGGTGGTAATGCCGGATG-3′) and Bif662-GC-r (5′-CCACCGTTACACC GGGAA-3′) was used to amplify V2-V4 of 16S rDNA of bifidobacteria (Satokari *et al*., 2001). The amplicons were separated by DGGE (Cleaver Scientific, UK). The DGGE gel containing 8% w/w acrylamide–bisacrylamide (37.5 : 1) with a denaturing gradient ranging

30-55% and 30-70% was used to separate the amplicons of LAB and bifidobacteria, respectively. The conditions for separation of PCR amplicons were 60 °C for 16 h with electrophoresis at constant voltage of 70 V and 85 V for LAB and bifidobacteria, respectively (Satokari *et al*., 2001). The DNA fragments were visualized by SYBR® Gold nucleic acid gel staining (Invitrogen, USA) and viewed by UV transillumination (Alpha innotech corporation, USA). The interesting bands were cut under UltraBright LED Transilluminator (Gellex, Taiwan) and purified by NucleoSpin® Extraction Π (Germany). The purified DGGE bands were sequenced at BioDesign Co. Ltd. (Bangkok, Thailand).

7.4 Results

7.4.1 Effect of probiotic, prebiotic and synbiotic supplements on the dominant fecal microflora in small scale with challenge system

FISH analysis was used to quantify the level of the dominant fecal microflora including *Bifidobacterium* sp., *Bacteriodes* group, *Lactobacillus*/ *Enterococcus* spp., *Clostridium histolyticum* group, *Eubacterium* in system supplemented with probiotic, prebiotic and challenged with *Sal.* Typhimurium SA2093. High number of *Salmonella* infection (>10^7 CFU g^{-1}) did not affect on the levels of the dominant fecal bacteria. There was little or no difference in total bacteria count in the *Salmonella* challenged and the control treatments. The supplementation of probiotic, saba extract, saba starch, the combination of probiotic and saba extract or probiotic and saba starch significantly impacted on the dominant members of human fecal microflora. Although, the level of total bacteria was similar in most treatments in the beginning of fermentation and continuously decreased until the end of fermentation (Figure 25), total bacteria count and eubacteria increased at 24 h of fermentation in the treatment challenged with *Sal.* Typhimurium SA2093 with the addition of probiotic and saba extract. Moreover, *Lactobacillus*/ *Enterococcus* spp. and *Bifidobacterium* were not significantly affected by *Salmonella* infection. During fermentation period, *Bifidobacterium* was significantly enhanced at 6 h, and dropped rapidly at 12 h of fermentation in the presence of saba extract and the combination of probiotic and saba extract. *Bifidobacterium* population remained consistent throughout the fermentation period in the treatments supplemented with the saba starch and the synbiotic of *L. plantarum* CIF17AN2 and the saba starch. *L. plantarum* CIF17AN2

supplementation resulted to significantly increase level of *Lactobacillus*/*Enterococcus* spp. group in all treatments indicating that this probiotic strain was able to maintain its viability and was able to compete with fecal microflora throughout fermentation period. Surprisingly, in treatment supplemented with synbiotic of *L. plantarum* CIF17AN2 and saba starch, the level of *Lactobacillus*/*Enterococcus* spp. group was stable throughout 12 h of fermentation thereafter significantly increased till the end of fermentation. Whereas, *Lactobacillus*/*Enterococcus* spp. group remained constant in most treatments without probiotic supplement except the addition of saba extract. Significant decrease of *Clostridium* population was observed in the treatment supplemented with both synbiotic formulae after 12 h of fermentation. About 2 log reduction in *Clostridium* count was detected in these treatments. However, increasing in *Clostridium* level was found in treatment supplemented with saba extract. *Clostridium* count reached to 8.15 log cell ml^{-1} at 24 h of fermentation. *Salmonella* challenge did not affect the level of *Bacteriodes*, neither did probiotic, prebiotic nor synbiotic supplements.

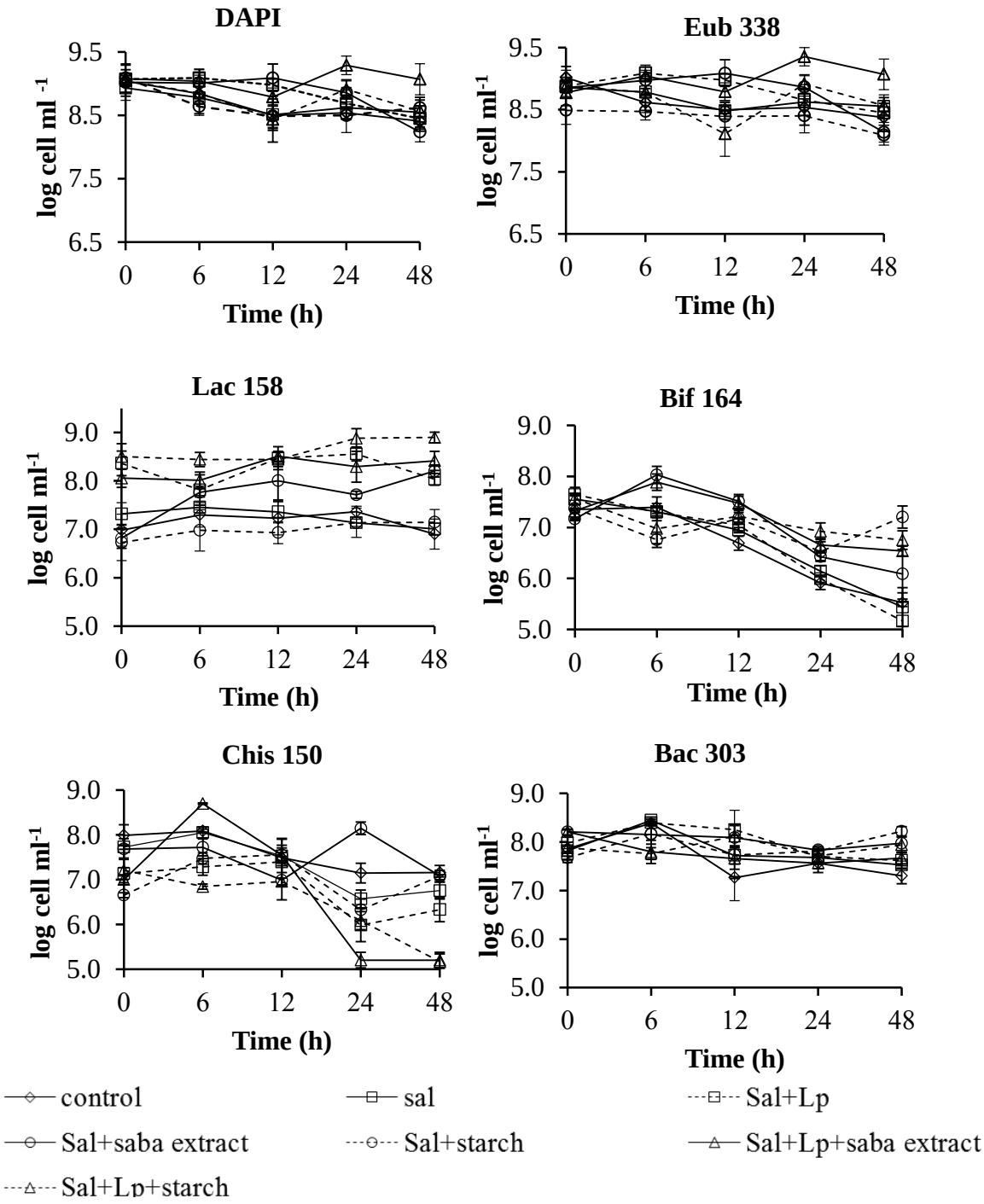


Figure 25. Changes of total fecal bacteria (DAPI), *Eubacterium* (Eub 338), *Lactobacillus*/*Enterococcus* spp. (Lac 158), *Bifidobacterium* (Bif 164), *Clostridium* (Chis 150) and *Bacteriodes* (Bac 303) in system supplemented with the combination of *L. plantarum* CIF17AN2 with either saba extract or saba starch in un-controlled pH batch culture system**.**

7.4.2 Effect of probiotic, prebiotic and synbiotic supplements on growth of *Sal. enterica* subsp. *enterica* serovar Typhimurium SA2093 challenged in the batch mixed culture system

The challenge test was conducted to reveal the effect of saba starch, saba extract, and synbiotic supplement (the combination of *L. plantarum* CIF17AN2 with either saba extract or saba starch) on the inhibition against the challenged *Sal.* Typhimurium SA2093. *Salmonella* count was significantly affected by the supplementations of prebiotic and synbiotics (Figure 26). Especially the presence of saba extract both with and without probiotic combination. *Salmonella* count was rapidly decreased and reached a non-detectable level within 24 h and 36 h in synbiotic combination of *L. plantarum* CIF17AN2 and saba extract and saba extract alone, respectively. Similarly, *Salmonella* count was also diminished in the presence of saba starch. At 48 h, *Salmonella* count reduced from 7.7 to 3.53 and 4.16 log CFU ml^{-1} when the synbiotic combination of *L. plantarum* CIF17AN2 with saba starch and the prebiotic of saba starch were added, respectively. However, the inhibition was not observed when only probiotic was added. The results indicated that prebiotic supplement was more influential to control *Salmonella* in the mixed culture of human fecal microflora than probiotic supplement. *Salmonella* level remained quite constant throughout 36 h and then a bit reduced at the end of fermentation in probiotic supplement as well as control, in which there was no any addition of any supplement. This indicated that *Salmonella* was not inhibited by adding only a probiotic of *L. plantarum* CIF17AN2. *Salmonella* was not detected in the negative control, in which *Sal.* Typhimurium SA2093 was not challenged confirming that there was no *Salmonella* contamination in the original feces. Therefore the number drawn from plate count represented the level of the challenged *Sal.* Typhimurium SA2093. The reduction of *Salmonella* in all systems generally corresponded to the decrease of pH (Figure 27). Such correlation was pronounced particularly in all treatments supplemented with prebiotics of either saba starch or saba extract. Decrease of pH could be due to the specific enhancement of saba starch or saba extract toward bifidobacteria and LAB population as shown in Figure 25.

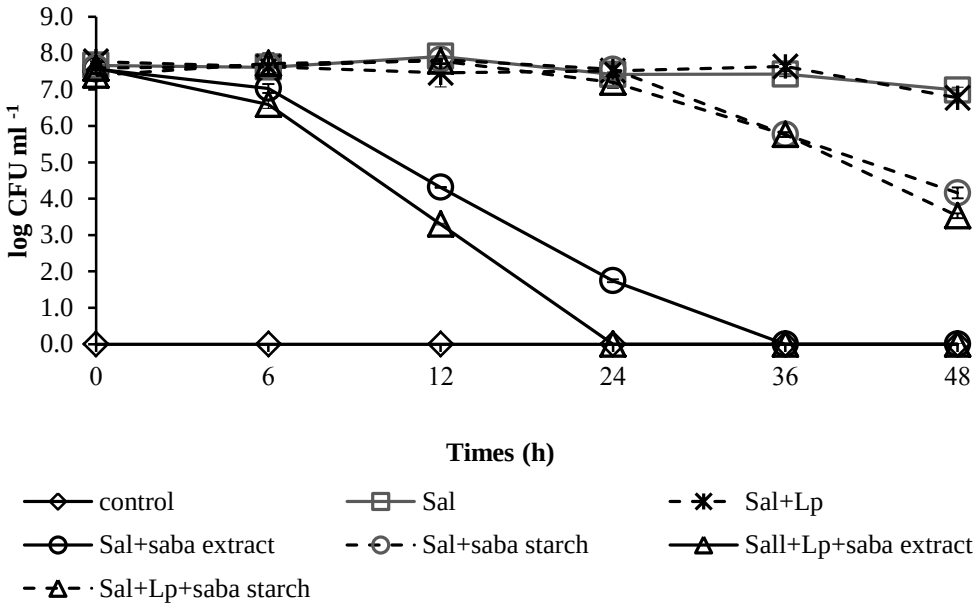


Figure 26. Growth of *Sal*. *enterica* subsp. *enterica* serovar Typhimurium SA2093 in system supplemented with the combination of *L. plantarum* CIF17AN2 with either saba extract or saba starch in un-controlled pH batch culture system.

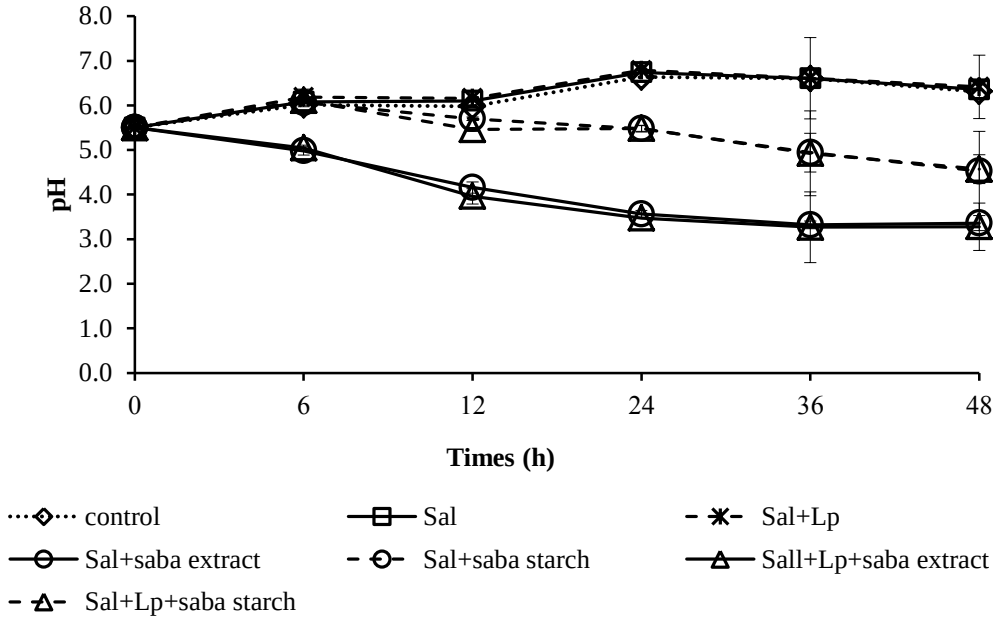


Figure 27. Changes of pH in system supplemented with the combination of *L. plantarum* CIF17AN2 with either saba extract or saba starch.

7.4.3 Effect of the synbiotic supplement on dominant fecal microflora in the challenged system under simulated human proximal colon condition (pH control).

The effect of selected prebiotic (saba starch) and synbiotic combination of *L. plantarum* CIF17AN2 and saba starch on dominant gut microflora in the system mimicked proximal region of human gastrointestinal tract (pH 5.5) was investigated. FISH analysis was performed to quantitatively determine the dominant members of human fecal microflora. The synbiotic supplement was effective impact on dominant gut microflora. Total bacteria count essentially remained stable until 12 h of fermentation in all treatments and started increasing in synbiotic and prebiotic supplements (Figure 28). At 24 h of fermentation more than 9 log cell ml^{-1} of total bacteria count was detected in saba starch and synbiotic treatments. Oppositely, slight decrease of total bacteria was found in the control once approaching without any supplements till the end of fermentation. In the same way, there was no change of *Eubacterium* level at 12 h of fermentation in all but the rapid increase was observed in saba starch and synbiotic treatments, whereas *Eubacterium* decrease was noted in the control. In addition, saba starch and synbiotic supplements led to noticeable dominant in *Bifidobacterium*. However, the synbiotic supplementation contributed to significantly higher increase of *Bifidobacterium* count than the prebiotic one. Similarly, lactobacilli level increased significantly in the synbiotic treatment compared to prebiotic addition and control system. Especially, at 24 h of fermentation, *Lactobacillu*s number in synbiotic treatment was more than 2 log cell ml^{-1} higher than other treatments. Nevertheless, the limitation of available substrate in batch system caused to diminish in *Lactobacillus* count after 24 h of fermentation. *Bacteriodes* was stable throughout the fermentation period when saba starch and synbiotic were supplemented. In contrast, *Bacteriodes* level was slightly decreased till the end of fermentation in the control system. No *Clostridium* growth supporting was detected in saba starch and synbiotic. Nearly 1 log reduction of *Clostridium* was found in both treatments at the end of fermentation. While, the reduction in *Clostridium* counts in control was less than 0.4 log cell ml^{-1}.

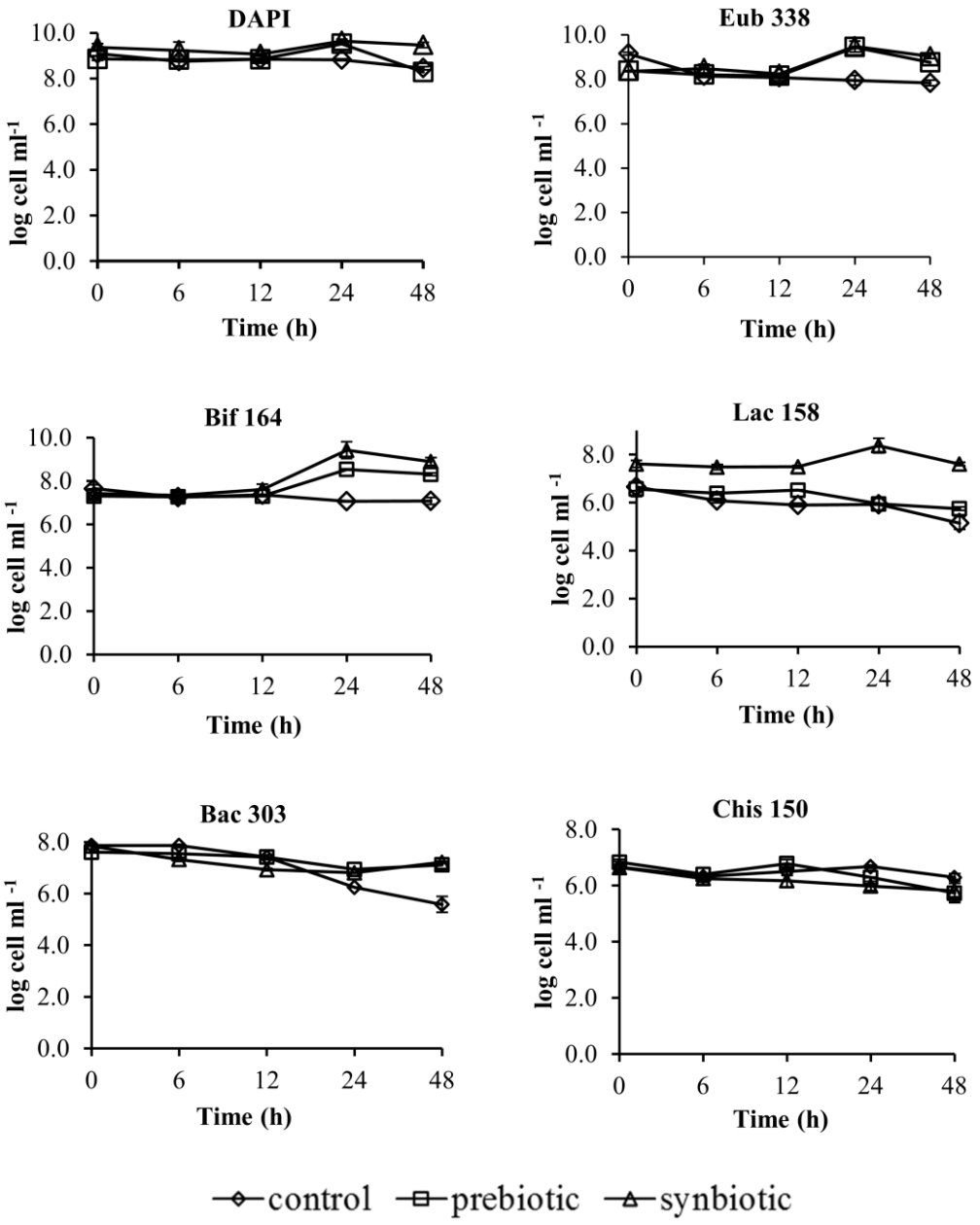


Figure 28. Effect of synbiotic supplement (*L. plantarum* CIF17AN2+saba starch) on total fecal bacteria (DAPI), *Eubacterium* (Eub 338), *Lactobacillus*/*Enterococcus* spp. (Lac 158), *Bifidobacterium* (Bif 164), *Clostridium* (Chis 150) and *Bacteriodes* (Bac 303) in *in vitro* pH-controlled (5.5) batch culture fermentation under simulated human proximal colon condition.

7.4.4 Effect of the synbiotic supplement on growth of *Sal.* Typhimurium SA2093 in the challenged system under simulated human proximal colon condition (pH control)

The *Salmonella* level was significantly affected by the supplementation of saba starch and synbiotic (Figure 29). At 48 h of fermentation, two log reductions of *Sal*. Typhimurium SA2093 was observed under simulated human proximal colon condition with pH control at 5.5. Whereas, there was only little or no change of *Salmonella* count in the control without the prebiotic and synbiotic supplementation.

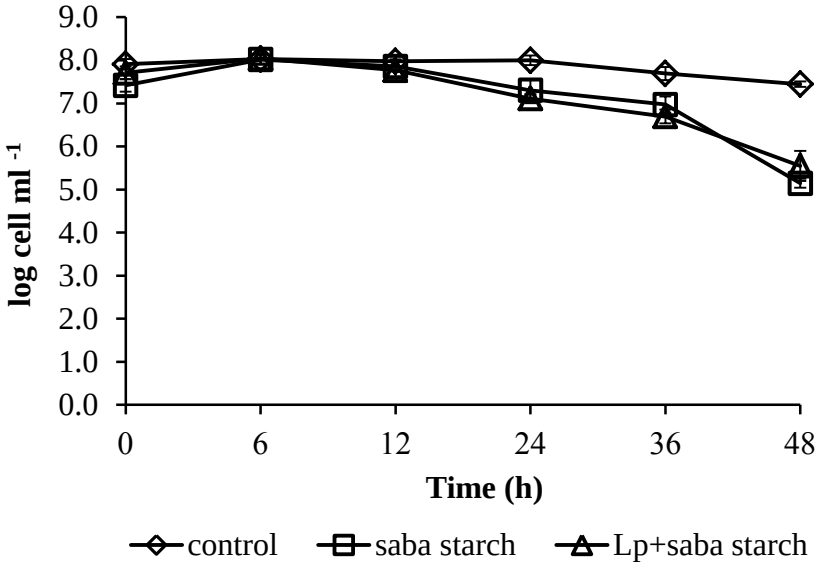


Figure 29. Growth of *Salmonella* Typhimurium SA2093 in the absence (control) and the presence of saba starch, and synbiotic (combination of saba starch and *L. plantarum* CIF17AN2) under the condition mimic human proximal colon with the competition to human fecal microflora.

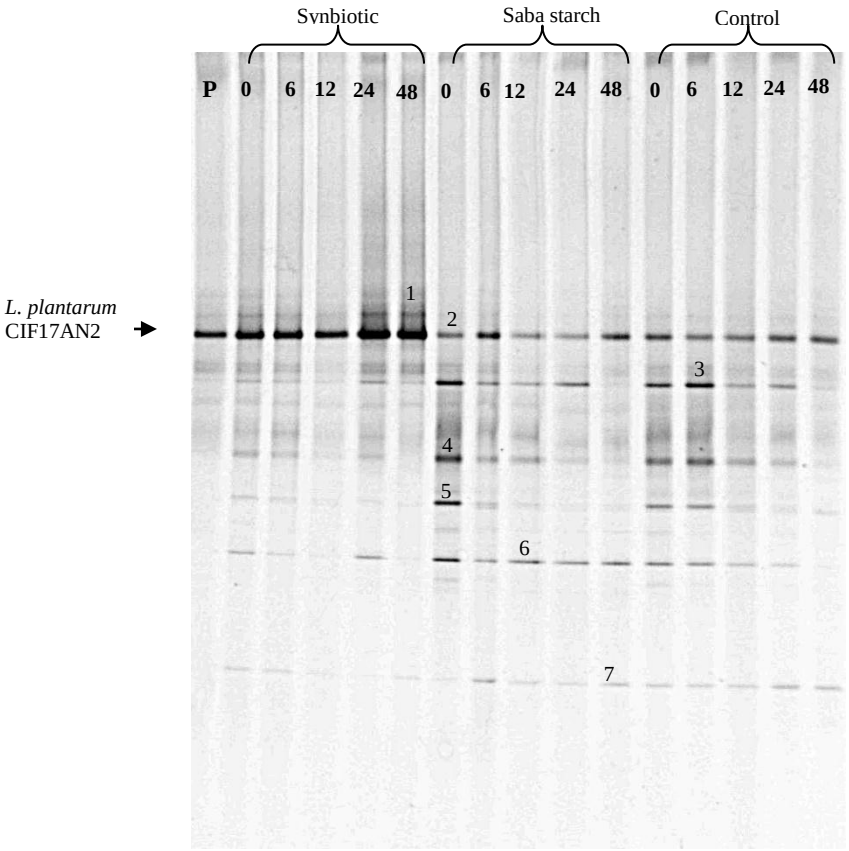
Table 25. Short chain fatty acids (from triplicate analysis) produced by fecal microflora in the pH-controlled batch culture fermentation challenged with *Sal*. Typhimurium SA2093 under simulated proximal colon fermentation system supplemented with prebiotic (saba starch) and synbiotic (*L. plantarum* CIF17AN2 + saba starch).

Time (h)	Short chain fatty acid concentration (mM)											
	Control				Prebiotic supplement				Synbiotic supplement			
	lactate	acetate	propionate	butyrate	lactate	acetate	propionate	butyrate	lactate	acetate	propionate	butyrate
0	0.00±0.00	0.02±0.01	0.07±0.01	0.03±0.04	0.32±0.01	0.42±0.24	0.59±0.10	0.90±0.11	0.75±0.26	0.08±0.03	0.36±0.29	0.43±0.25
12	0.00±0.00	1.09±0.36	0.50±0.02	0.53±0.01	5.70±0.05	14.49±1.04	2.89±0.02	2.31±0.01	4.89±0.24	16.11±1.32	3.29±0.11	2.580.10
24	0.05±0.01	3.09±0.55	2.46±0.02	1.24±0.02	6.78±0.21	20.04±0.39	7.74±0.17	3.92±0.13	7.87±0.48	22.39±1.07	6.77±0.09	2.22±0.08
48	0.04±0.01	8.52±0.01	3.41±0.06	1.87±0.03	10.91±1.64	25.57±0.48	10.16±0.70	6.93±0.52	10.42±1.84	27.24±0.73	12.25±0.71	4.74±0.71

7.4.5 Bifidobacteria and lactic acid bacteria profile in pH-controlled batch culture fermentation

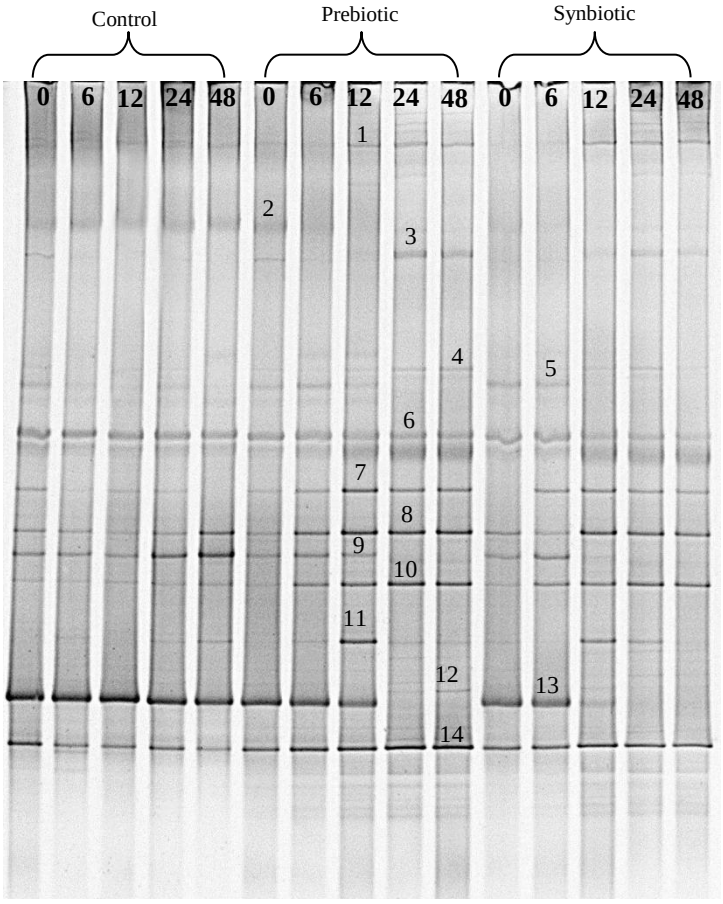
PCR-DGGE was performed to analyze the changing of gut lactic acid bacteria and bifidobacteria in pH-controlled batch culture fermentation with and without prebiotic or synbiotic supplementation. According to DGGE analysis, no difference in lactic acid bacteria population was observed among control, prebiotic and synbiotic treatments (Figure 30). Although, decreasing in band intensity was found in some treatments, they still maintained. In addition, *L. plantarum* (Band 2) was presented in all treatments. High band intensity of *L. plantarum* was found in synbiotic supplement system especially at the end of fermentation.

With nested-PCR-DGGE approach, diversity of bifidobacteria in each treatment was revealed (Figure 31). The DGGE profile demonstrated that prebiotic and synbiotic supplementation modified bifidobacterial profile distinctively different from the control. The major bands that persisted throughout the whole period of fermentation (band 6, 8 and 14) corresponded to *Bifidobacterium aldolescentis*. The presence of band 12 only in synbiotic and prebiotic treatments and increase intensity of band 7, 8 and 10 in synbiotic and prebiotic treatments confirmed prebiotic effect of saba starch and synbiotic effect of *L. plantarum* CIF17AN2 and saba starch in supporting the growth of bifidobacteria in the *in vitro* simulated human proximal colon. However, there was not different in band richness and band intensity of bifidobateria population between prebiotic and synbiotic supplements indicating that *L. plantarum* CIF17AN2 supplement did not alter bifidobacteria population in the system. The loss of band 2, 9 and 13 in prebiotic and synbiotic added treatments showed that prebiotic saba starch and synbiotic (saba starch and *L. plantarum* CIF17AN2 were selectively supporting the growth of only specific group of bifidobacteria.



Band	Closest relative	Accession number	% Similarity
L1	*Lactobacillus brevis* strain JNB3	JX218942	100
L2	*Lactobacillus plantarum* strain MJ0301	JX099893	100
L3	*Lactobacillus* sp. NBRC 107227	AB682526	100
L4	*Weissella confusa* strain MNC39	JQ754469	100
L5	*Leuconostoc lactis*	AB596941	100
L6	*Lactobacillus ruminis* ATCC 27782	CP003032	99
L7	Uncultured Firmicutes bacterium clone N4-1	GU955906	99

Figure 30. DGGE profile exhibiting diversity of lactic acid bacteria in the pH-controlled (5.5) batch culture fermentation under simulated proximal colon of human gastrointestinal tract supplemented with synbiotic (*L. plantarum* CIF17AN2 + saba starch).



Band	Closest relative	Accession number	% Similarity
B1	*Bifidobacterium* sp. G29	HM626174	97
B2	*Bifidobacterium longum* subsp. *longum* KACC 91563	CP002794	99
B3	*Bifidobacterium* sp. M7	HM626175	98
B4	*Bifidobacterium* sp. TM-7	AB218972	98
B5	*Bifidobacterium longum* subsp. *infantis* 157F	AP010890	100
B6	*Bifidobacterium adolescentis* strain SaLPYG-3	JN093131	99
B7	*Bifidobacterium longum* DJO10A	CP000605	100
B8	*Bifidobacterium adolescentis* ATCC 15703	AP009256	99
B9	*Bifidobacterium adolescentis* strain BBMN23	GQ380694	99
B10	*Bifidobacterium adolescentis* strain H1	JN020353	100
B11	*Bifidobacterium* sp. HMLN14	JF519687	99
B12	Uncultured *Bifidobacterium* sp	AB490507	100
B13	*Bifidobacterium adolescentis* strain LCR4	HQ259739	98
B14	*Bifidobacterium adolescentis*	HE974924	100

Figure 31. DGGE profile exhibiting diversity of bifidobacteria in the pH-controlled (5.5) batch culture fermentation under simulated proximal colon of human gastrointestinal tract supplemented with developed synbiotic (*L. plantarum* CIF17AN2 + saba starch).

7.5 Discussion

Saba starch was studied for its efficiency to support the growth of beneficial human gut microflora in *in vitro* small scale un-controlled pH batch culture fermentation. In addition, we also studied the potency of saba starch on challenged *Salmonella*. Saba extract was used as control. Saba starch exerted potential prebiotic characteristic by supporting the growth of beneficial bacteria like lactobacilli and bifidobacteria which are claim to be health promoting bacteria (Lavermicocca *et al*., 2005). In addition the combination of saba starch and *L. plantarum* CIF17AN2 also showed positive effect on gut microflora. Especially, on lactobacilli population, significant higher lactobacilli count was found in synbiotic combination of saba starch and *L. plantarum* CIF17AN2 than synbiotic combination of saba extract and *L. plantarum* CIF17AN2. In addition, the combination of saba starch with *L. plantarum* CIF17AN2 also prevented the growth of less favorable bacteria like clostridia. As well, the challenged *Salmonella* was inhibited in saba starch with and without combination of *L. plantarum* CIF17AN2 treatments. However, the anti-*Salmonella* activity in treatments presented of saba extract was higher than in treatments presented of saba starch. The antimicrobial activity in these treatments maybe came from dropping of pH in culture medium to about 3.3 and 4.5 in saba extract and saba starch, respectively.

Normally, the target of prebiotic and synbiotic was the large bowel (Macfarlane *et al.,* 2006) which is known to be a heterogenous environment and the pH in this area was 5.5-6.8 (Sannasiddappa *et al*., 2011). In addition, there are some evidences indicating that large intestine is primary site involvement during severe and/or fatal enteric infections with *Salmonella* (zu Bentrup *et al.,* 2006). Therefore, the system simulated proximal region of human gastrointestinal tract was set up in order to study the effect of saba starch and developed synbiotic product (combination of saba starch and *L. plantarum* CIF17AN2) on the survival of challenged *Sal*. Typhimurium SA2093 and growth of dominant gut microflora in realistic condition.

As the same pattern with un-controlled pH system, saba starch and synbiotic significantly enhanced growth of bifidobacteria and lactobacilli which are associated with various health benefits at the cellular systemic levels. Such effects are considered to include the inhibition of pathogenic microorganisms, antimutagenic and anticarcinogenic activity,

increase of the immune response, and reduction of cholesterol levels (Du Toit *et al*., 1998). Moreover, SCFAs are the main metabolites of these probiotic bacteria. They are an important source of energy for the human gut and can be transported to other tissue and organs of the human body (Kedia *et al.,* 2009). Acetate is primarily used in muscle tissue, whereas propionate is mainly used by the liver. Butyrate is the most important energy source for colonocytes, which should be constantly supplied to maintain good health (Kedia *et al.,* 2009). Saba starch and synbiotic product did not support the growth of clostridia in contrast the number of clostridia decreased in these treatments. The lack of growth enhancement in clostridia was a beneficial characteristic of synbiotic and saba starch. This is because clostridia generally produce various metabolic end products, which maybe contribute to the progression of colorectal cancer and inflammation bowl disease (Bialonska *et al*., 2010). Although the saba starch and synbiotic did not support the growth of *Bacteriodes*, they did not have any negative effect on this ubiquitous bacteria.

The reduction of *Salmonella* in system simulated proximal human large intestine in prebiotic and synbiotic treatments indicated that the antibacterial activity caused by either synbiotic effect of *L. plantarum* CIF17AN2 and saba starch or the prebiotic effect of saba starch that supported the growth of beneficial fecal bacteria (LAB/Bifidobacteria). Prebiotic addition could lead to production of many antimicrobial compounds apart from low pH effect. Short chain fatty acids (SCFA) are major bacterial fermentation products. Up to 95% of SCFA (acetate, propionate and butyrate) produced during carbohydrate fermentation (Tuohy *et al.,* 2005), which correlated to this study. The majority of short chain fatty acids were investigated in the prebiotic and synbiotic treatments. The result was shown in Table 25. The beneficial effects of resistance starch on large-bowel function have been reported that appear to be exert through SCFA formed by bacterial fermentation (Topping *et al*., 2003). The undissociated form of organic acids can easily penetrate the lipid membrane of the bacterial cell and once internalized into the neutral pH of the cell cytoplasm dissociated into anions and protons. Export of excess protons requires consumption of cellular adenosine triphosphate (ATP) and may result in depletion of cellular energy. The antimicrobial activity of *n*-butyric acid against *Salmonella* Typhimurim and *Clostridium perfringens* has been reported (Namkung *et al.,* 2011). In addition, the undissociated short-chain fatty acids produced by the colon flora inhibiting the growth of *Pseudomonas aeroginosa* have also been reported (Levison, 1973).

The DGGE profile revealed that human isolate probiotic bacteria were able to survive and grow in the condition simulated human proximal colon. This finding is correlative to the result drawn from FISH analysis according to *Lactobacillus/Enterococcus* spp. count. However, prebiotic and synbiotic supplementation did not distinctly change in the overall of lactic acid bacteria population in *in vitro* human proximal colon. In accordance with another study, GanedenBC^{30} supplementation did not elicit major changes in the microbiota *in vitro* when they were determined using FISH technique. Nevertheless the microbial diversity of control and GanedenBC^{30} supplementation systems was somewhat divergent (Honda *et al.,* 2011). Due to starch owes much of its functionality to two major high molecular-weight carbohydrate component, amylose and amylopectin as well as to the physical organization of these macromolecules into the granular structure resulted to few bacterial species able to utilize this carbohydrate as carbon source (Bello-Pérez *et al*., 1999). This is in agreement with the report of Gopal and colleague (2001), *Lactoba cillus rhamnosus* DR20 prefers sugars with a lower degree of polymerization, i.e, disaccharides and monosaccharide. In contrast, we found that prebiotic and synbiotic supplementation modified bifidobacterial population. The bifidobacteria profile in supplemented treatments was distinctively different from the control. However, the increasing of band intensity and the richness of bifidobacteria almost came from prebiotic action of saba starch. Harmsen and colleague (2002) have been reported the prebiotic effect of inulin in increase bifidobacterial numbers in the gut without changing the species composition, whilst the probiotic had almost no effect. However, the selective supporting the growth of one or limited number of saba starch resulted to dominant in specific number of bifidobacteria this led to the absence of some bifidobaceria band in supplemented system. The dominance of other species in gut microflora influenced the detection limit of DGGE by affecting both the efficiency of DNA extraction and the PCR amplification due to the possible competition among templates (Ercolini, 2004; Fontana *et al.,* 2005).

7.6 Conclusion

Saba starch and the combination of saba starch and *L. plantarum* CIF17AN2 exerted prebiotic and synbiotic effect through supporting the growth of beneficial bacteria in human gastrointestinal tract in both pH-controlled and un-controlled pH batch culture fermentation system with no effect or suppression the growth of less desirable bacteria. In

addition, their prebiotic and synbiotic effect resulted to inhibit the growth of challenged *Sal*. Typhimurium SA 2093. Saba starch and synbiotic supplementation in simulated proximal region human gastrointestinal tract did not modify lactic acid bacteria profile whereas, bifidobacteria population was dramatically changed.

CHAPTER 8

SUMMARY

1. Lactic acid bacteria (313 strains) and bifidobacteria (17 strains) were isolated from 25 fecal samples. After screening probiotic properties, only 7 strains of LAB identified as *L. casei* (NIF1A7 and NIF7AN12), *L. plantarum* (CIF17A2, CIF17A4, CIF17A5, CIF17AN2 and CIF17AN8) and 5 strains of bifidobacteria identified as *B. longum* subsp. *longum* (NIF3AN3 and NIF7AN2) and *B. bifidum* (NIF7AN3, NIF7AN5 and NIF7AN10) displayed potential probiotic properties. These isolated strains were able to survive after exposure to condition simulated gastrointestinal tract and also able to inhibit the growth of both Gram-positive and Gram-negative pathogenic bacteria.

2. Human isolates *L. casei* (NIF1A7 and NIF1AN12), *L. plantarum* (CIF17A2, CIF17A4, CIF17A5, CIF17AN2 and CIF17AN8), *B. longum* subsp. *longum* (NIF3AN3 and NIF7AN2) and *B. bifidum* (NIF7AN3, NIF7AN5 and NIF7AN10) were susceptible to ampicillin, penicillin, erythromycin, tetracycline and rifampicin but seemed to intrinsic resistance to vancomycin and polymyxin B.

3. Our lactobacilli and bifidobacteria isolated from infant feces were able to adhere to mucosal mucin. *B. bifidum* and *L. casei* were strong mucin adhesive probiotic bacteria whereas *L. plantarum* and *B. longum* subsp. *longum* were intermediate mucin adhesive strains. Adhesion ability of these bacteria still maintain after passed through simulated condition in upper gastrointestinal tract. In addition, mucin adhesive lactobacilli and bifidobacteria were able to inhibit the adhesion of enteropathogens to mucin by competitive adhesion. Bacterial surface protein was extremely involved in adhesion of high mucin adhesive *B. bifidum*. The physicochemical surface characteristic of this high mucin adhesive bacterium was strong hydrophobicity and electron donor properties.

4. Four plants extract including saba, gros michel, mung bean and okra exerted prebiotic characteristic by supporting the growth of probiotic bacteria. Among 4 of them, saba extract showed the best prebiotic properties. This prebiotic extract not only supported the growth of probiotic bacteria but also enhanced the antimicrobial activity of

potential probiotic bacteria both in broth microdilution assay in 96-well plate and co-culture system especially, when it combined with *L. plantarum* CIF17AN2 and *L. plantarum* CIF17AN8. Both combinations displayed potential synbiotic by reducing rapidly the level of *S. aureus* TISTR 1466, *E. coli* TISTR 780 and *Sal.* Typhimurium SA2093 until they become undetectable within 24 h. However, only the combination of saba extract and *L. plantarum* CIF17AN2 exerted properly synbiotic effect. This combination supported the growth of beneficial gut microflora and diminished the growth or had no positive effect on deleterious bacteria like clostria and bacteroides. However, saba extract showed undesirable prebiotic property due to it supporting the growth of clostridia group when it presented alone.

5. Drying under vacuum oven gave the highest probiotic survivability. Saba starch was a perfect fiber for protection *L. plantarum* CIF17AN2 during vacuum drying process. It helped probiotic bacteria maintaining its viability during 8 weeks storage at refrigerated and unrefrigerated temperature. Saba starch could not be able to protect dried-synbiotic product from low pH and bile salt conditions.

6. Saba starch and synbiotic combination of saba starch and *L. plantarum* CIF17AN2 exerted prebiotic and synbiotic effect through supporting the growth of beneficial bacteria in human gastrointestinal tract in both pH-controlled and un-controlled pH batch culture fermentation system with no effect or suppression the growth of less desirable bacteria. In addition, their prebiotic and synbiotic effect resulted to inhibit the growth of challenged *Sal*. Typhimurium SA 2093. Saba starch and synbiotic supplementation in simulated proximal region human gastrointestinal tract did not modify lactic acid bacteria profile whereas, bifidobacteria population was dramatically changed.

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APPENDIX

APPENDIX

Preparation of culture medium

1. MRS agar (de Man Rogosa and Sharp)

Composition per liter:

Proteose peptone	10.0 g
Beef extract	10.0 g
Yeast extract	5.00 g
Dextrose	20.0 g
Polysorbate 80	1.00 g
Ammonium citrate	2.00 g
Sodium acetate	5.00 g
Magnesium sulphate	0.10 g
Manganese sulphate	0.05 g
Dipotassium phosphate	2.00 g
Agar	12.0 g

Preparation

Suspend 67.15 grams in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Distribute in tubes, bottles or flasks as desired. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

2. Mueller Hinton broth (MHB)

Composition per liter:

Beef extract	3.00 g
Casein acid hydrolysate	17.0 g
Starch	1.50 g

Preparation

Suspend 22 grams in 1000 ml distilled water. Heat if necessary to dissolve the medium completely. Dispense and sterilize by autoclaving at 115-121°C for 10 minute.

3. Xylose-Lysine Deoxycholate Agar (XLD Agar)

Composition per liter:

Yeast extract	3.00 g
L-Lysine	5.00 g
Lactose	7.50 g
Sucrose	7.50 g
Xylose	3.50 g
Sodium chloride	5.00 g
Sodium deoxycholate	2.50 g
Sodium thiosulphate	6.80 g
Ferric ammonium citrate	0.80 g
Phenol red	0.08 g
Agar	15.0 g

Preparation

Suspend 56.68 grams in 1000 ml distilled water. Heat with frequent agitation until the medium boils. DO NOT AUTOCLAVE OR OVERHEAT. Transfer immediately to a water bath at 50°C. After cooling, pour into sterile Petri plates

4. Minimal medium

Composition per liter :

Peptone water	2.00 g
Yeast extract	2.00 g
NaCl	0.10 g
K_2HPO_4	0.04 g
KH_2PO_4	0.04 g
$MgSO_4.7H_2O$	0.01 g
$CaCl_2.6H_2O$	0.01 g
$NaHCO_3$	2.00 g
Cysteine.HCl	0.50 g
Bile salts	0.50 g
Tween 80	2.00 g

Preparation

Add components to distilled water and bring volume to 1 liter. Mix thoroughly and distribute to injection vial, sterile for 15 min at 121°C.

5. Basal medium

Composition per liter :

Peptone water	2.00 g
Yeast extract	2.00 g
NaCl	0.10 g
K_2HPO_4	0.04 g
KH_2PO_4	0.04 g
$MgSO_4.7H_2O$	0.01 g
$CaCl_2.6H_2O$	0.01 g
$NaHCO_3$	2.00 g
Hemin	0.05 g
(Dissolved in a few drops of 1 mol/l NaOH)	
Cysteine.HCl	0.50 g
Bile salts	0.50 g
Tween 80	2.00 g
Vitamin K_1	10.0 µl

Preparatiom of medium:

Add components to distilled water and bring volume to 1 liter. Mix thoroughly and distribute to injection vial, sterile for 15 min at 121°C.

VITAE

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List of Publication and Proceeding

Publications

Uraipan, S. and Hongpattarakere, T. Diversity and antagonistic characteristics against foodborne pathogens of probiotic lactic acid bacteria and bifidobacteria isolated from feces of healthy thai infants. (Submitted manuscript).

Uraipan, S. and Hongpattarakere, T. Bifidogenic characteristic and protective effect of saba starch on survival of *Lactobacillus plantarum* CIF17AN2 during vacuum-drying and storage. (Submitted manuscript).

Uraipan, S. and Hongpattarakere, T. Synbiotic effect of saba starch and *Lactobacillus plantarum* CIF17AN2 on growth of challenged *Salmonella* Typhimurium SA2093 in mixed culture system (Manuscript preparation).

Proceeding

Uraipan, S. and Hongpattarakere, T. 2010. Antimicrobial susceptibility of lactic acid bacteria and bifidobacteria from infant feces. The 1st Current Drug Development International Conference. May 6-8, 2010 Woraburi Phuket Resort & Spa, Phuket, Thailand, pp. 119-122.