การหาปริมาณและคุณสมบัติในการยับยั้งเชื้อก่อโรค ของไบฟีโดแบคทีเรียและแลคโตบาซิลไล ในอุจจาระของเด็กทารกที่เลี้ยงด้วยนมมารดาและเลี้ยงด้วยนมผสม

นางสาวชุติมา จิตตประสาทศีล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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QUANTIFICATION AND DETERMINATION OF ANTAGONISTIC ACTIVITY OF BIFIDOBACTERIA AND LACTOBACILLI IN FAECES OF BREAST-FED AND MIXED-FED INFANTS

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Thesis Title	QUANTIFICATION AND DETERMINATION OF ANTAGONISTIC ACTIVITY OF BIFIDOBACTERIA AND LACTOBACILLI IN FAECES OF BREAST-FED AND MIXED-FED INFANTS
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ชุติมา จิตตประสาทศิล : การหาปริมาณและคุณสมบัติในการยับยั้งเชื้อก่อโรค ของไบฟิโดแบคทีเรีย และแลคโตบาซิลไล ในอุจจาระของเด็กทารกที่เลี้ยงด้วยนมมารดาและเลี้ยงด้วยนมผสม. (QUANTIFICATION AND DETERMINATION OF ANTAGONISTIC ACTIVITY OF BIFIDOBACTERIA AND LACTOBACILLI IN FAECES OF BREAST-FED AND MIXED-FED INFANTS) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : รศ. ดร. สมหญิง ธัมวาสร, 113 หน้า.

งานวิจัยครั้งนี้มีวัตถุประสงค์เพื่อศึกษาผลกระทบของการเลี้ยงทารกด้วยนมมารดาหรือนมผสม ต่อปริมาณเชื้อไบฟิโดแบคทีเรียและแลคโตบาซิลไลในเด็กทารก และศึกษาความสามารถของเชื้อที่แยกได้ในการยับยั้งเชื้อก่อโรคในระบบทางเดินอาหาร ได้แก่ enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), *Salmonella* Typhimurium, *Shigella flexneri* และ *Vibrio cholerae*

ผลการหาปริมาณของไบฟิโดแบคทีเรียและแลคโตบาซิลไล ในเด็กทารกที่เลี้ยงด้วยนมมารดาเพียงอย่างเดียวจำนวน 30 ราย และเด็กทารกที่เลี้ยงด้วยนมผสม (นมมารดาร่วมกับนมผง) จำนวน 33 ราย โดยวิธี real-time quantitative PCR พบว่าจำนวนไบฟิโดแบคทีเรียในเด็กทารกที่เลี้ยงด้วยนมมารดาและเลี้ยงด้วยนมผสมเท่ากับ 8.26 และ 8.42 log16S rRNA genes/g ตามลำดับ ($p = 0.67$) และจำนวนแลคโตบาซิลไลในเด็กทารกทั้งสองกลุ่มเท่ากับ 6.84 และ 7.20 log16S rRNA genes/g ตามลำดับ ($p = 0.14$) ซึ่งเชื้อทั้งสองชนิดมีปริมาณไม่แตกต่างกันอย่างมีนัยสำคัญในเด็กทารกทั้งสองกลุ่ม ทำการเพาะแยกเชื้อไบฟิโดแบคทีเรียและแลคโตบาซิลไลจากอุจจาระของเด็กทารก และคัดเลือกมาทำการทดสอบความสามารถในการยับยั้งเชื้อก่อโรคในระบบทางเดินอาหารโดยวิธี agar spot method ทำการทดสอบเชื้อไบฟิโดแบคทีเรีย 111 สายพันธุ์ พบว่า 106 สายพันธุ์ สามารถยับยั้งการเจริญของเชื้อก่อโรคได้ โดยเฉพาะ 10 สายพันธุ์ มีปฏิกิริยาอย่างชัดเจน เกิดเป็นโซนใสต่อเชื้อ *V. cholerae* และ *Sh. flexneri* และเป็นโซนบางๆ ต่อเชื้อ EHEC, EPEC, ETEC, EIEC และ *S.* Typhimurium การทดสอบเชื้อแลคโตบาซิลไล 39 สายพันธุ์ พบว่า 14 สายพันธุ์ สามารถยับยั้งการเจริญของเชื้อก่อโรคได้ โดยมี 7 สายพันธุ์ เกิดเป็นโซนใสขนาดเล็กต่อเชื้อ *V. cholerae* การวิเคราะห์สปีชีส์ของเชื้อไบฟิโดแบคทีเรียและแลคโตบาซิลไล โดยการหาลำดับเบสของดีเอ็นเอ พบว่าเชื้อที่แยกได้จากเด็กทารกไทยอายุ 1 เดือน ได้แก่ *B. longum*, *B. bifidum*, *B. adolescentis*, *B. pseudocatenulatum*, *L. gasseri*, *L. salivarius*, *L. fermentum*, *L. ruminis*, *L. mucosae*, *L. vaginalis*, *L. oris*, *L. rhamnosus* และ *L. casei* ตามลำดับ

จากผลการศึกษานี้ การเลี้ยงเด็กทารกด้วยนมมารดาหรือนมมารดาร่วมกับนมผงไม่มีผลต่อปริมาณของเชื้อไบฟิโดแบคทีเรียและแลคโตบาซิลไลในเด็กทารกไทย เชื้อไบฟิโดแบคทีเรียที่เพาะแยกได้มีความสามารถในการยับยั้งเชื้อก่อโรคมากกว่าแลคโตบาซิลไล ทั้งในแง่ของความหลากหลายของเชื้อก่อโรคและความรุนแรงในการยับยั้ง เชื้อไบฟิโดแบคทีเรียที่มีความสามารถมากที่สุดนี้มีความเหมือนกับ *B. adolescentis* เพียง 90% แสดงว่าอาจเป็นเชื้อสายพันธุ์ใหม่ ซึ่งเหมาะสมสำหรับการศึกษาชนิดและคุณสมบัติของเชื้อต่อไป

สาขาวิชาจุลชีววิทยาทางการแพทย์ ลายมือชื่อนิสิต .....ชุติมา จิตตประสาทศิล.....

ปีการศึกษา 2551 ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

4889070520 : MAJOR MEDICAL MICROBIOLOGY
KEYWORDS : INFANTS / BIFIDOBACTERIA / LACTOBACILLI / PROBIOTICS / REAL-TIME PCR / QUANTIFICATION / ANTAGONISTIC ACTIVITY

CHUTIMA JITTAPRASATSIN : QUANTIFICATION AND DETERMINATION OF ANTAGONISTIC ACTIVITY OF BIFIDOBACTERIA AND LACTOBACILLI IN FAECES OF BREAST-FED AND MIXED-FED INFANTS. ADVISOR : ASSOC. PROF. SOMYING TUMWASORN, Ph.D., 113 pp.

This study aimed to determine the effect of feeding method on the quantity of bifidobacteria and lactobacilli in infants and investigate the antagonistic activities of these bacteria against enteric pathogens such as enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), *Salmonella* Typhimurium, *Shigella flexneri* and *Vibrio cholerae*.

The bifidobacteria and lactobacilli counts were compared between the 30 exclusively breast-fed and 33 mixed-fed infants (infants who received breast milk and formula milk) by real-time, quantitative PCR assays. The number of bifidobacteria in breast-fed and mixed-fed infants (mean values 8.26 and 8.42 log16S rRNA genes/g wet weight faeces, respectively; $p = 0.67$) and lactobacilli in these two groups (mean values 6.84 and 7.20 log16S rRNA genes/g respectively; $p = 0.14$) were not significantly different. One hundred and eleven *Bifidobacterium* and 39 *Lactobacillus* isolates from infants faeces were tested for their antagonistic activities against seven gastrointestinal pathogens by agar spot method. One hundred and six isolates of *Bifidobacterium* had inhibitory activities. Interestingly, 10 *Bifidobacterium* isolates demonstrated strong antagonistic activities with clear zones against *V. cholerae* and *Sh. flexneri* and weak activities against EHEC, EPEC, ETEC, EIEC and *S.* Typhimurium. Fourteen isolates of *Lactobacillus* had inhibitory activities, 7 isolates demonstrated weak antagonistic activities against *V. cholerae* with small clear zone. Genotypic identification by DNA sequencing demonstrated that the *Bifidobacterium* and *Lactobacillus* species in faeces of Thai infants at the age of one month included *B. longum*, *B. bifidum*, *B. adolescentis*, *B. pseudocatenulatum* and *L. gasseri*, *L. salivarius*, *L. fermentum*, *L. ruminis*, *L. mucosae*, *L. vaginalis*, *L. oris*, *L. rhamnosus* and *L. casei*, respectively.

Breast feeding and mixed feeding did not have effect on the number of bifidobacteria and lactobacilli in Thai infants. *Bifidobacterium* isolates possessed stronger antagonistic activities than *Lactobacillus* isolates in term of the varieties of inhibited enteric pathogens and the magnitude of inhibition. *Bifidobacterium* isolates with the most potent antagonistic activities had 90% DNA similarity with the most matched *B. adolescentis*. This indicated that these isolates might be new strains or new species and thus the potential sources of novel probiotic strains.

Field of Study : Medical Microbiology Student's Signature : Chutima Jittaprasatsin.
Academic Year : 2008 Advisor's Signature : Somying Tumwasorn

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CONTENTS

LIST OF TABLES

Table **Page**

LIST OF FIGURES

LIST OF ABBREVIATIONS

CFU	Colony forming unit
H_2O_2	Hydrogen peroxide
CO_2	Carbon dioxide
rRNA	Ribosomal RNA
EDTA	Ethylenediamine tetraacetic acid
PCR	Polymerase chain reaction
MRS	deMan Rogosa Sharp
ATCC	American type culture collection
BHI	Brain heart infusion agar
OD	Optical density
mM	millimole
mm	millimetre
hr	hour
min	minute
s	second
^oC	Degree celsius
µl	microliter
ng	nanogram
µg	Microgram
ml	Millilitre
mg	milligram
SD	Standard deviation
bp	Base pair
dNTP	Dideoxynucleotide tri-phosphate
WHO	World Health Organization
MC	Modified Columbia medium
hr	hour

DMST	Department of Medical Science Thailand

CHAPTER I

INTRODUCTION

The gastrointestinal tract consists of an extremely complex microbiota, which has many physiological functions such as a barrier effect to protect the digestive tract against the pathogenic bacteria, stimulation of the immune system and bioconversion of the nutrients[1,2]. The gastrointestinal tract is sterile until birth and microbial colonization begins immediately after delivery. The subsequent development of gut microflora during infancy is a complex succession of bacterial species, influenced by factors like gestational age (premature or full term), mode of delivery (vaginal or caesarean section), hygiene and antibiotic use, geographical zone and type of feeding (breast or formula) [1,2]. *Bifidobacterium* and *Lactobacillus* species are frequently implicated to have health-promoting effects in the human and animal intestinal tract. Their probiotic effects have been reported to inhibit pathogenic bacteria, reduce colon cancer, increase the immune response, and decrease serum cholesterol[3]. In addition, it has also been reported that they produce antimicrobial substances or bacteriocins that inhibit undesirable pathogens causing diarrhea or other disease in the human intestine[4].

It has been suggested that the composition and properties of human milk (lower content and different composition of proteins, lower phosphorus content, large variety of oligosaccharides, numerous humoral and cellular mediators) can enhance the development of an infant gut microflora dominated by bifidobacteria and lactobacilli [4]. Critical reviews of published data obtained by conventional culture

techniques suggest no marked differences between breast-fed and formula-fed groups in the counts of bifidobacteria or *Bacteroides* spp. and in the levels of lactobacilli, with the main differences being focused on the higher numbers and isolation frequencies of enterococci and clostridia, the greater levels of enterobacteria and the lower counts of staphylococci in formula-fed infants[1,2]. Recently, studies based on molecular methods have confirmed the differences in the establishment of lactic acid bacteria and bifidobacteria according to feeding regime[5], whereas others suggest similar prevalence and counts of *Bifidobacterium* spp. in both feeding groups. Little information is available on the bacterial microflora of children fed a mixture of breast and formula milk.[7]

For decades, the quantification of bacterial genera and species present in the intestinal microbiota was based on traditional bacteriological culture and biochemical identification techniques. However, these methods are time-consuming and are limited by low sensitivities, the inability to detect non-culturable bacteria as well as unknown species, and the low levels of reproducibility due to the multitude of species to be identified and quantified[7]. To overcome these drawbacks, culture-independent molecular methods based on 16S rRNA genes such as fluorescent in situ hybridization [21], dot-blot hybridization with rRNA-targeted probes[22], denaturing gradient gel electrophoresis[23], temperature gradient gel electrophoresis[24], and cloning and sequencing of rDNA[25] have been introduced to obtain a better understanding of the gut microbiota. Although these rRNA or rDNA - based techniques offer a feasible alternative to conventional culture-based methodologies and provide valuable information on the GI tract microbial community, it is apparent that they are not

optimal for quantitative evaluation of complex microbial ecosystems because of their low sensitivity and inconveniency for accurate quantification.

Real –time PCR is a powerful advancement of the basic PCR technique and it has been successfully applied for quantification of bacterial DNA in various environments such as faeces, colonic tissue, gastric tissue and periodontal samples.[28]

The present study aimed at the quantification of bifidobateria and lactobacilli in breast-fed infants and formula-fed infants by real-time PCR and the investigation of the antagonistic activities against enteric pathogens of bifidobateria and lactobacilli isolated from the infant faeces.

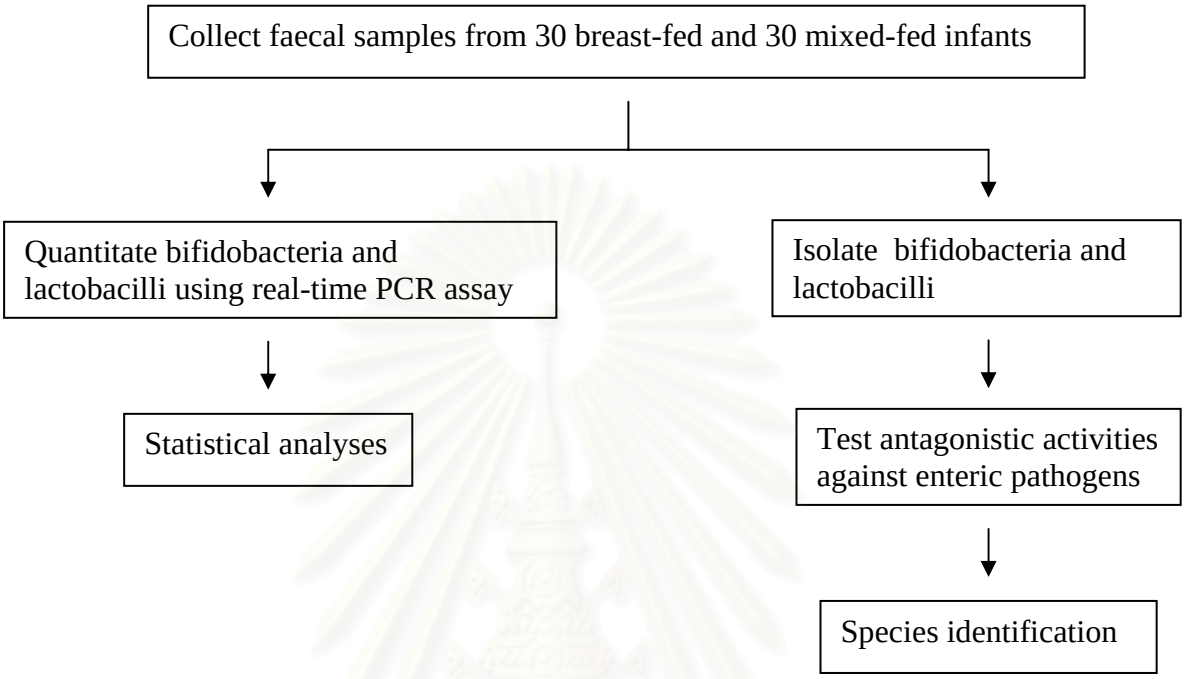
Hypothesis

1. Type of infant feeding influences the number of bifidobacteria and lactobacilli in infant faeces
2. Thai healthy infants are potential sources of bifidobacteria and lactobacilli with antagonistic activities against enteric pathogens

Objectives

1. To quantitate bifidobacteria and lactobacilli in the faeces of breast-fed and formula-fed infants using real-time PCR assay.
2. To isolate bifidobacteria and lactobacilli from faeces of breast-fed and mixed-fed infants and test their antagonistic activities against enteric pathogens.

Flow Chart of Conceptual Framework



Expected Outcome

1. Find the effect of type of infant feeding on the number of bifidobacteria and lactobacilli in infant faeces
2. Find bifidobacteria and lactobacilli with antagonistic activities against enteric pathogens

CHAPTER II

LITERATURE REVIEWS

1. The human intestinal microbiota

The human large intestine is a densely populated microbial ecosystem. Several hundred species of bacteria are usually present and the total weight of microbiota living within the colonic lumen is estimated to be several hundred grams.1 There are up to 10^{13}–10^{14} total bacteria in the human intestinal tract, i.e. 10- to 20-fold more than the total number of tissue cells in the entire body.2 Most of the bacteria are obligate anaerobes, including clostridia, eubacteria, bacteroides groups and the genus bifidobacterium, such as *Bifidobacterium bifidum* and *Bifidobacterium infantis*. *Bifidobacterium* is a member of the dominant microbiota (i.e. $>10^{8}$–10^{9} colony forming unit (CFU)/g using culture methods, >1% of the total bacteria count using molecular biology methods), both in human faeces (3.2% ± 0.55 of total bacterial rRNA) and in the content of the caecal lumen (5.2 ± 0.37%) as shown by culture and molecular hybridization using rRNA-targeted probes or quantitative PCR.3–6

It is a long-standing belief, which probably originated with Metchnikoff at the turn of the 20th century, that some gut bacteria are beneficial to health, whilst others may be harmful. Obviously, some gut bacteria are harmful in that they produce toxins causing diarrhoea, mucosal invasion and activation of carcinogens is self-evident. Such bacteria are thought to include some *Clostridium* spp., sulphate-reducing and amino acidfermenting species. The main potentially health-enhancing bacteria are the bifidobacteria and lactobacilli, both of which belong to the lactic acid bacteria (LAB)

group.7 These two genera do not include any significant pathogenic species and their dominance in the faeces of breast-fed babies is thought to impart protection against infection.8, 9 The health interest of the *Bifidobacterium* genus is reflected in the commonly-accepted definition of prebiotics: food ingredients that selectively stimulate the growth and activity of bacteria in the gut, usually bifidobacteria (bifidogenic effect) and lactobacilli thus procuring health benefits.10, 11

2. *Bifidobacterium*

2.1 Bifidobacteria : Safety in use

The safe use of bifidobacteria is supported by the long historical consumption of fermented milks and the growing knowledge about bifidobacteria taxonomy and physiology.12, 13 Lactic acid-producing bacteria in foods are considered as commensal microorganisms with little or no pathogenic potential.14 Indeed, a recent review of the safety of lactobacilli and bifidobacteria used as probiotics concluded that they posed no health risks for consumers.15

Regarding taxonomy, modern molecular techniques, including polymerase chain reaction-based and other genotyping methods, have become increasingly important for species identification and for the differentiation of bifidobacteria strains.16

‘16S rRNA sequence analysis (usually used to produce phylogenic trees) is not suitable to distinguish different species of *Bifidobacterium*.17 So, the gene sequence of heat-shock protein of 60 kDa (HSP 60) is preferentially used;

furthermore, it is found as a single copy in almost all bacterial species. The phylogenic tree is realized comparing a DNA fragment of 0.6 kb of the HSP 60 of each studied *Bifidobacterium* species.

It should be noted that this recognition of the safety of such strains will be formalized in a European regulatory framework that is in the process of defining the criteria to be evaluated when assessing the safety of microorganisms used in the food and feed industry.18

2.2 Physiological effects and clinical benefits of bifidobacteria

The results of the main human and animal studies carried out to further elucidate the physiological effects of *B. animalis* strain DN-173 010 and to assess their clinical pertinence

Colonic fermentation

Through fermentation, bacterial growth is stimulated (biomass), and organic acids (lactic acid and short chain fatty acids-SCFAs), are produced together with gases: H_2, CO_2 and CH_4. Lactic acid is produced by many gut bacterial species, mainly bifidobacteria and lactobacilli. SCFAs (mainly acetate, propionate and butyrate) are the major end-products of bacterial fermentative reactions in the colon and the principal anions in the human hindgut.34 All SCFAs are rapidly absorbed from the hindgut and stimulate salt and water absorption. They are then metabolized, principally by the gut epithelium, liver and muscle. One of their major properties is

their trophic effect on the intestinal epithelium. Moreover, butyrate, a most interesting SCFA, is an important energy source for the colonic epithelium and regulates cell growth and differentiation.35–37 Even if bifidobacteria do not produce butyrate directly, they produce lactate that may be transformed in butyrate.38 Butyrate has been shown to reduce the rate of transformed cell growth, in a concentration-dependent manner, and to promote expression of differentiation markers in vitro, thus leading to cells reversion from a neoplastic to a non-neoplastic phenotype.37

In addition to fermentation products, gut bacteria, including bifidobacteria are able to synthesize vitamins, especially B vitamins.39, 40 No in vivo data concerning the production of B vitamins by bifidobacteria and its impact on B vitamins status in humans is available at the present time.

Barrier effects

A number of mechanisms by which probiotics may protect the host from potentially harmful entities have been proposed, e.g. production of inhibitory substances, blockade of adhesion sites and stimulation ofimmunity.41

Production of inhibitory substances. *Bifidobacterium infantis* strain has been shown to exert a broad spectrum of antimicrobial properties through production of antimicrobial compounds, unrelated to acid production, which inhibit the growth of pathogens.42 In other studies, the activity of bifidobacteria strains in vitro was shown to result from antimicrobial compounds present in the spent culture supernatants, suggesting that the compounds were secreted.41 Interestingly, Fujiwara et al.43 recently described a protein factor produced by *Bifidobacterium longum* SBT 2928,

with a molecular weight of at least 100 000, which inhibited adhesion of enterotoxigenic *Escherichia coli* strain Pb176 which expresses colonization factor adhesion II, to the gangliotetrasylceramide GA1 molecule in vitro. Two strains of bifidobacteria were found to produce an antibacterial lipophilic factor (or several factors) with an estimated molecular weight of <3500.41

Blockade of adhesion sites. Probiotics may prevent infection by out-competing with pathogenic viruses or bacteria for binding sites on epithelial cells.44–46 In a study using human Caco-2 cell cultures, *B. animalis* DN-173010 demonstrated adhesion properties to human cells, even when EGTA was added to the medium: this confirms that adhesion of this *Bifidobacterium* strain to intestinal cells is not calcium-dependent. Further investigation on this strain showed that no extra cellular protein factor is required for its adhesion (A. Servin, personal communication).

Stimulation of immunity. In experimental conditions, *B. longum* increases the immunological and defensive functions of germ free mice.47–49 *Bifidobacterium breve* YIT4064 enhances antigen specific IgA-antibody directed against rotavirus in the mouse.50 The barrier effect generated by some probiotics may derive from positive modulation of the mucous layer that separates the intestinal lumen from the colonocytes. Indeed, probiotics may change the gut mucosal barrier by stabilizing the intestinal mucosa, normalizing intestinal permeability and improving gut immunology, leading to the prevention of the overgrowth of pathogenic bacteria and viruses.50, 51 Much work remains to be done to specify the mechanisms of action of particular probiotics against particular pathogens and to show the translation of these mechanisms into human benefits.

Effects of bifidobacteria on gastrointestinal disease

Infectious diarrhoea. Acute infections of the gut are usually self-limiting and characterized by diarrhea and, often, vomiting. The principal pathogens are viruses and bacteria. Considering the absence or small number of studies specifically relating to bifidobacteria alone in this section, clinical trials involving mixed preparation of probiotics have been introduced.

Diarrhoea because of rotavirus infection.Rotavirus is the most common cause of acute childhood diarrhoea. Many clinical studies evaluated the effect of probiotics on rotavirus-associated acute diarrhoea, especially in children. Saavedra et al. conducted a double-blind, placebo-controlled trial. Fifty-five hospitalized infants who were randomized to receive a standard infant formula or the same formula supplemented with *B. bifidum* (later renamed *B. lactis*) and Streptococcus thermophilus.62 During the 17 months of follow up, 31% of the patients given the standard infant formula, but only 7% of those receiving the probiotic supplemented formula developed diarrhoea. The prevalence of rotavirus shedding was significantly lower in the infants receiving the probiotic supplemented formula.62 This effect was confirmed in a prospective study including 175 children. The study showed that those receiving bifidobacteria-supplemented milk-based formula were protected against symptomatic rotavirus infection.63 The prophylactic effect were recently confirmed in a multi-centre, double-blind, controlled trial involving 90 infants aged <8 months who lived in residential nurseries or foster care centres. The study evaluated the efficacy of a milk formula supplemented with viable *B. lactis* strain Bb 12 in terms of the prevention of acute diarrhoea. The number of days with diarrhoea and the daily

probability of diarrhoea were significantly reduced in the probiotic group (1.15 ± 2.5 and 0.84 days) vs. the conventional formula group (2.3 ± 4.5 and 1.55 days).64 Feeding infants with *B. lactis* reduced their risk of contracting diarrhoea 1.9-fold (range,1.33–2.6).65

Antibiotic-associated diarrhoea. Diarrhoea caused by thegrowth of pathogenic bacteria is the most common side effect of antibiotic use. Probiotics may inhibit this growth by releasing inhibitory substances or bacteriocins, as has been demonstrated with some strains in vitro.59, 66, 67 To date, the main probiotics used are *Lactobacillus* GG, *Enterococcus* SF68 and *Saccharomyces* boulardii.68 One double-blind placebo-controlled study of 10 adults tested the effects of a daily consumption of 3 cups/day of *B. longum* yoghurts on erythromycinassociated gastrointestinal effects.69 Faecal weight, stool frequency, and abdominal complaints were significantly increased when erythromycin was given with placebo yoghurt but not when *B. longum* yoghurts were being taken. Moreover the simultaneous intake of *B. longum* yoghurts with erythromycin induced a sharp fall in clostridia spore count, suggesting that these yoghurts could reduce antibiotic-associated alterations in the intestinal microflora. In another study, subjects receiving a mix of prebiotics (fructooligosaccharides) and probiotics (including *B. longum* BB 536) during oral administration of cefpodoxime proxetil twice daily were shown to be less susceptible to *Clostridium difficile* colonization than subjects receiving prebiotics only orplacebo.69 These results were confirmed in a recent double-blind, placebo-controlled study investigating the role of a probiotic containing both *Lactobacillus* and *Bifidobacterium* in the prevention of *C. difficile*-associated diarrhoea. The study was conducted on 150 elderly patients receiving antibiotic therapy and randomized to

receive the treatment for 20 days. For the patients developing diarrhoea, the incidence of samples positive for *C. difficile*-associated toxins was 2.9% in the probiotic group vs. 7.25% in the placebo-control group. When specimens from all patients were tested, 46% of probiotic patients were *C. difficile* toxin-positive vs.78% in the placebo group.70

Bifidobacteria, as probiotics, may become an important means of enhancing digestive health and preventing disease. In order to realize this potential fully, research must focus on the following areas: (i) identification of *Bifidobacterium* strains that can withstand gastrointestinal transit (i.e. gastric acidity, bile salts and Paneth cell secretions); (ii) identification of the *Bifidobacterium* species and strains that are effective against specific disease processes or in disease prevention; (iii) investigation of the mechanisms of probiotic action; and (iv) development of new association between bifidobacteria strains and prebiotics. Currently, the utilization of probiotics and prebiotics is an interesting field of research as several probiotic strains, including *B. animalis* DN-173 010, show a more preferential fermentation pattern when associated with short-chain oligomers than with monomers.119–121 A recent study79 has shown a beneficial effect of a prebiotic and probiotic association highlighting the growing interest of synbiotics in digestive health.

3. General Characteristics of *Lactobacillus*

3.1 Biology of *Lactobacillus*

Lactobacilli are gram positive, catalase negative, non-spore forming rods or coccobacilli varying from long and slender, sometimes bent rods to short rods, often coryneform, chain formation is common. Some strains exhibited bipolar bodies, internal granulation or a barred appearance with the Gram reaction or methylene blue stain [(1)]. They are members of the lactic acid bacteria, a broadly defined group characterized by the formation of lactic acid as a sole or main end product of carbohydrate metabolism [(50)].

Colonies of lactobacilli on agar media are usually small (2-5 mm), with entire margins, convex, smooth, glistening and opaque without pigment. In rare cases, they are yellowish or reddish. Some species form rough colonies [(1)]. Lactobacilli do not develop characteristic odors when grown in common media. However, they contribute to the flavor of fermented food by producing various volatile compounds, such as diacetyl and its derivatives and even H_2S and amines in cheese [(1)].

Lactobacilli are extremely fastidious organisms which adapted to complex organic substrates. They require not only carbohydrates as energy and carbon source, but also nucleotides, amino acid and vitamins. The various requirements for essential nutrients are normally met when the media contain fermentable carbohydrate, peptone, meat and yeast extract. Supplementations with tomato juice, manganese, acetate and oleic acid esters, especially Tween 80, are stimulatory or even essential for the most species. Therefore, these compounds are included in the widely used MRS medium [(51)]. With glucose as a carbon source, lactobacilli may be either

homofermentative, producing more than 80% lactic acid as main product or heterofermentative, producing mixed products of lactic acid, carbon dioxide, ethanol and/ or acetic acid in equimolar amounts [50, 52]. The lactic acid formed by the various fermentation pathways possesses either L-or the D-configuration depending on the stereospecificity of the lactate dehydrogenase present in the cells [1].

Lactobacilli grow best in slightly acidic media with and initial pH of 4.5-6.4. Most strains are fairy aerotolerant while optimal growth is achieved under microaerophilic or anaerobic conditions. Increased CO_2 concentration (~5%) may stimulate growth. Most lactobacilli grow best at mesophilic temperature with an upper limit around 40°C [1]. Lactobacilli should be incubated in jars evacuated and filled with 90% N_2 or H_2 plus 10% CO_2 or in anaerobic jars using H_2 plus CO_2 generating kits.

3.2 Ecology of *Lactobacillus*

Lactobacilli grow under anaerobic conditions or at least under reduced oxygen tension in all habitats providing carbohydrates, proteins and nucleic acids and vitamins. A mesophilic to slightly thermophilic temperature range is favorable. Lactobacilli are generally acidophilic. They decrease the pH of their substrate by lactic acid formation to below pH 4.0, thus preventing growth of other competitors except other lactic acid bacteria and yeasts. These properties make lactobacilli valuable inhabitants of the intestinal tract of humans and animals and important contributors to food technology. Many species of *Lactobacillus* are microbiota of humans and animals.

Lactobacilli occur in nature in low numbers at all plant surfaces and together with other lactic acid bacteria, grow luxuriously in all decaying plant material, especially decaying fruits. Thus, lactobacilli are important for the production as well as the spoilage of fermented vegetable feed and food and beverages. Most species isolated have been: *L. plantarum, L. brevis, L. coryneformis, L. casei, L. curvatus, L. sake* and *L. fermentum* [53-56].

In milk and dairy products, milk contains no lactobacilli when it leaves the udder, but becomes very easily contaminated with lactobacilli by dust and dairy containers. After prolonged incubation bacteria take over, due to their higher acid tolerance [1]. *L. delbrueckii* subsp. *bulgaricus* is a component of the well known yogurt microbiota [57].

Lactobacilli play an important role during the processing of fermented sausages containing added sucrose. Various species of lactobacilli multiply during cold storage of meat products. This process delays spoilage by proteolytic bacteria. The most common naturally occurring species found in ripening raw sausages are *L. plantarum, L. brevis, L. farciminis, L. alimentarius, L. sake* and *L. curvatus* [56, 58].

3.3 *Lactobacillus* of the human digestive tract

Lactobacillus species are one of the most commonly found gram-positive bacteria in the human microbiota. The different numbers and species of lactobacilli depend on the genetic background of host as well as their age and health [59]. Lactobacilli have been found in the oral cavity, gastrointestinal tract, vaginal and breast milk [1, 60, 61]. The *Lactobacillus* of gastrointestinal system consists of various species, subspecies and the most frequently occurring lactobacilli belong to six

species: *L. acidophilus, L. salivarius, L. casei, L. plantarum, L. fermentum* and *L. brevis* [59]. In addition, the frequently occurrence of *L. reuteri* in the gastrointestinal tract of humans and animal has also been detected [1, 5, 62].

In the oral cavity, a wide range of *Lactobacillus* species have been found. These species are *L. acidophilus, L. salivarius, L. casei, L. rhamnosus, L. plantarum, L. fermentum, L. cellobiosus, L. buchneri,* and *L. brevis* [59].

Lactobacillus species could be detected in all parts of the human gastrointestinal tract including the stomach [62], which is characterized by a pH of 2.2–4.2. Relatively few bacterial species can tolerate these acidic conditions and most organisms ingested with food and saliva are killed by the hydrochloric acid, reducing the population to about 10^3 CFU/ml, containing mainly lactobacilli and streptococci [44, 63]. In the duodenum and jejunum, lactobacilli and enterococci are the dominant bacteria [44, 64, 65]. The microbiota becomes more complex in the ileum, being qualitatively similar to that of the large intestine and the relative proportion of lactobacilli decreases. *Lactobacillus* species can be cultured from human feces at counts varying from none to $<10^9$ CFU/gm feces [64, 66, 67]. Tannock, et al. investigated the succession of lactobacilli in feces of 10 human subjects during a period of fifteen months. They found that the dominant and persistent species were *L. ruminis, L. salivarius, L. acidophilus, L. crispatus* and *L. gasseri* which were regularly detected in the feces [67]. *L. ruminis* was also detected as the predominant species over several months and *L. salivarius, L. acidophilus, L. crispatus* and *L. gasseri* could be detected regularly [68, 69]. *L. reuteri* has been rarely detected in human fecal samples in recent studies either by culture or by nucleic acid-based methods [70-71]. Furthermore, these studies indicated that lactobacilli such as *L. paracasei,*

L. rhamnosus, L. delbrueckii, L. brevis, L. johnsonii, L. plantarum and *L. fermentum* are rather transient, persist for limited times, or in undetectable low numbers that may increase in response to dietary factors or changes in the host's conditions [62].

The presence of the lactobacilli in the digestive tract has historically been considered as beneficial to the host. At the beginning of the last century, Elie Metchnikoff (1845–1916) stated that toxic substances produced by members of the intestinal microbiota are absorbed from the intestinal tract and contribute to the aging process [71]. Microbes capable of degrading proteins, releasing ammonia, amines and indole were considered harmful and bacteria like lactobacilli (which ferment carbohydrates to obtain energy and have little proteolytic activity) were thought to be beneficial [72, 73]. Lactobacilli are considered to benefit the health of the consumer when ingested as probiotics [64, 74]. *Lactobacillus* species commonly detected in the intestine (fecal samples), oral cavity and associated with food and probiotics products were shown in Table 1.

Table 1. *Lactobacillus* species commonly detected in the intestine (fecal samples), oral cavity and associated with food and probiotics products [68].

Species	Oral Cavity	Feces	Food	Probiotics
L. crispatus	+	+	-	+
L. gasseri	+	+	-	+
L. reuteri	-	+	+	+
L. ruminis	-	+	-	-
L. salivarius	+	+	-	+
L. acidophilus	+	+	-	+
L. brevis	+	+	+	-
L. casei	+	+	+	+
L. delbrueckii	-	+	+	+
L. fermentum	+	+	+	+
L. johnsonii	-	+	-	+
L. paracasei	+	+	+	+
L. plantarum	+	+	+	+
L. rhamnosus	+	+	+	+
L. sakei	+	-	+	-
L. curvatus	+	-	+	-

3.4 Antimicrobial compounds of *Lactobacillus*

The ability of lactic acid bacteria especially, *Lactobacillus* to produce the antimicrobial substances has historically long been used to preserve foods. They have ability to produce various antimicrobial substances which can be classified as low-molecular-mass (LMM) compounds such as organic acid (lactic acid, acetic acid and propionic acid), hydrogen peroxide (H_2O_2), diacetyl (2, 3-butanedione), uncharacterized compounds and high-molecular-mass(HMM) compounds like bacteriocins [(75)].

Organic acids

Lactic acid is produced by homofermentation or equimolar amounts of lactic acid, acetic acid, propionic acid, ethanol and carbon dioxide are produced by heterofermentation. Acetic acid is the strongest inhibitor and has a wide range of inhibitory activity, inhibiting yeasts, molds and bacteria [(76)] while propionic acid has been observed to exert a strong antimicrobial effect, in particular towards yeasts and molds [(77)]. Mixtures of lactic and acetic acids have been observed to reduce the growth rate of *S. enterica* ser. var. Typhimurium more than either acid alone, suggesting a synergistic activity [(78)].

Hydrogen peroxide

Hydrogen peroxide is produced in the presence of oxygen. The bactericidal effect of hydrogen peroxide (H_2O_2) has been attributed to its strong oxidizing effect on the bacterial cell. Also, some of the hydrogen peroxide producing reactions scavenges oxygen, thereby creating an anaerobic environment that is unfavorable for

certain organisms. It has been suggested that hydrogen peroxide production is particularly important for colonization of the urogenital tract by lactobacilli. Colonization by such lactobacilli has been found to decrease the acquisition of human immune deficiency virus (HIV) infection, gonorrhea and urinary tract infection [(79)].

Diacetyl

Diacetyl (2, 3-butanedione) is produced by citrate fermentation. It was identified as the aroma and flavor component in butter [(80)]. The antimicrobial activity of diacetyl has been documented since 1927 and was reviewed by Jay, J.M. [(80)]. This author found that this molecule is characterized by a broad antimicrobial activity at concentrations ranging between 200 and 1000 part per million (ppm). Gram-positive bacteria were more resistant, while gram-negative and yeasts exhibited a higher sensitivity to this molecule [(80)]. Diacetyl was able to strongly inhibit *Escherichia coli* O157:H7 and *Salmonella* Typhimurium at concentrations 50 ppm [(81)].

Reuterin

Reuterin, a low–molecular-mass compound, is produced by *L. reuteri*, a heterofermentative species and a member of microbiota of gastrointestinal tract of humans and animals. It is formed during the anaerobic growth on a mixture of glucose and glycerol by the action of glycerol dehydratase which catalyzes the conversion of glycerol into reuterin, 3-hydroxypropanal [(27)]. During log phase, no reuterin is produced since it is reduced by the reducing power from glucose metabolism. However, when cells enter stationary phase, reuterin starts to accumulate [(82)]. Although other bacteria also assimilate glycerol via the same pathway, accumulation

and excretion of reuterin appears to be a specific property of *L. reuteri* [83, 84]. Reuterin has a very broad spectrum of antimicrobial activity. It was found to have antibacterial, antifungal and antiprotozoal activity. Harmful organisms sensitive to reuterin include species of *Salmonella, Shigella, Vibrio*, enterohemorrhagic *E. coli*, enterotoxigenic *E. coli*, *Clostridium, Staphylococcus, Listeria, Candida* and *Trypanosoma* [85-88].

Bacteriocins

Bacteriocins are compounds produced by bacteria in order to inhibit the growth of other bacteria. Bacteriocins can be regarded as antibiotics, but their mode of action is different from many antibiotics. They have a narrow killing spectrum and thus they are generally able to kill only bacteria closely related to the producing strains [89].

Bacteriocins produced by *Lactobacillus* can be divided into four major classes: Class I-lantibiotics or bacteriocins which are small peptides (<5 kDa), containing unusual amino acids not normally found in nature; Class II-small hydrophobic bacteriocins which are heat-stable peptides (<13 kDa); Class III-large bacteriocins which are heat-labile proteins (>30 kDa); and Class IV-complex bacteriocins which are proteins with lipid and/or carbohydrate moieties. Class I and II bacteriocins are currently the main classes of bacteriocins due to their abundance and potential use in commercial applications [75]. Most of the bacteriocins produced by *Lactobacillus* spp. belong to the Class II bacteriocins. *L. plantarum* is most often associated with bactericin production. A number of bacteriocins have been isolated from various *L. plantarum* strains [90].

4. Methods for evaluation of antimicrobial activity

The agar diffusion method

Agar diffusion method has long been used for testing antimicrobial activity and is probably the most commonly used for detection of antimicrobial activity [(75)]. The method has been widely used for biologically derived compounds. It includes agar well diffusion assay [(91)] and disc assay [(92)]. In this test, an antimicrobial compound is applied to an agar plate on a paper disc or a well [(92)]. The compound diffuses into agar resulting in a concentration gradient that is inversely proportional to the distance from the disc or well. The size of inhibition zone around the disc or well is a measure of the degree of inhibition. The incubation conditions are dependent on the indicator organisms used. Since highly hydrophobic antimicrobial compounds cannot diffuse in agar, they are not suitable for tests by this method [(75)].

Several modified procedures based on the agar diffusion method have also been used for testing antimicrobial activity. These procedures include the agar spot method [(93)] and spot-on-lawn method [(94)].

The agar and broth dilution methods

Agar and broth dilution methods are suitable for microorganisms with variable growth rate and for anaerobic or microaerophilic microorganisms. The results are expressed as minimum inhibitory concentration (MIC), which is the lowest concentration of an antimicrobial that prevent growth of a microorganisms after a specific incubation period. In this method, an antimicrobial agent is serially diluted and a single concentration added to a culture tube or plate with nonselective broth or

melted agar medium, which is then inoculated with test organisms and incubated. The MIC is defined as the lowest concentration at which no growth occurs or absence of turbidity in a medium following incubation. The broth dilution assay has been used for the detection of the antimicrobial activity of reuterin produced by *L. reuteri* and the activity of reuterin was expressed as MIC values or the maximum dilutions of the reuterin fraction [27, 85].

New rapid screening methods for the detection of antimicrobial activity have been developed. In one method, indicator organisms are exposed to bacteriocins after staining with carboxyfluorescein diacetate [95]. Fluorescence is measured by flow cytometry and the effect of bacteriocin is seen as a decrease of fluorescence when the fluorescent compound leaks from the cells. Another method has used bioluminescent indicator strains in screening of antimicrobial activity. Luciferase genes are transformed into indicator strains and indicator strains start to produce light in reaction [96]. This method increases the sensitivity and allows for real-time assessment of antimicrobial activity [75].

5. *Bifidobacteria* and *Lactobacillus* as Probiotics

5.1 Background of probiotics

The first significant introduction of the probiotic concept was by Nobel Prize Laureate, Elie Metchnikoff, at the beginning of the 1900s. He believed that the complex microbial population in the colon was adversely affecting the host through so-called 'autointoxication', and reported that Bulgarian peasants, who consumed large quantities of fermented milk containing lactic acid bacteria were associated with good health and longevity [72]. The milk contained the microorganism "Bulgarican bacillus" which was later renamed *Lactobacillus bulgaricus*. Metchnikoff reasoned that these bacteria eliminated putrefactive bacteria from the gastrointestinal tract [97]. The works of Metchnikoff are regarded as the birth of probiotics [98]. This was attributed to the health-promoting values of the live organisms [90]. Subsequent research looked to confirm that the consumption of lactic acid bacteria was having a beneficial effect on health. In Japan, Shirota selected beneficial strains of lactic acid bacteria which could survive passage through the intestine, and subsequently used them to develop fermented milk drinks, known as *L. casei* Shirota in Yakult product [99]. It was soon established that there were many species of lactic acid bacteria in the intestine and these have subsequently been incorporated into many probiotic preparations [90]. Lactobacilli and bifidobacteria are the most frequently used genera as probiotics [100]. At present, probiotics products are available in variety of forms, including dairy foods, fermented milk, food supplements and dietary supplements and the range of products continued to expand. In parallel, the market for such foods

continues to develop, with most activity in developed countries, in particular in Europe, Japan and the United States [101].

5.2 Definition of probiotics

The term “probiotics” which comes from the Greek meaning “for life” was first used to described substances produced by one microorganism that stimulate the growth of another microorganism [102]. Fuller defined a probiotics as “a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance” [103]. Fuller’s definition has since been broadened to state that “a probiotic is a mono-or mixed culture of live microorganisms which, when applied to animal or man, affect the host beneficially by improving the properties of the indigenous microflora” [104]. Salminen et al. [105] proposed that probiotics be defined as microbial cell preparations or components of microbial cells that have a beneficial effect on the health and well-being of the host. A recent formal definition of probiotics was agreed by a working party of European scientists and is given as “a live microbial feed supplement that is beneficial to health” [106]. This emphasized the importance of definitive improvements in health. A probiotic effect can therefore be manifested via the gut microflora by ingestion of viable micro-organisms, either in the form of specific preparations such as powders, tablets or capsules, or through yoghurts and other fermented foods. They can contain only one, or several different species of microorganisms [90].

5.3 Probiotics properties

For the selection and assessment of potential probiotics, several research groups have recommended that a microorganism should have some predefined criteria in order to be considered as probiotics. These criteria were summarized in Table 2 [100, 107]. Strains of human origin are most suitable because some health promoting benefits may be species specific and microorganisms may perform optimally in the species from which they were isolated [108]. However, it is the specificity of the action, not the source of the microorganism that is recognized as being most important when selecting probiotics strains for particular applications [109]. All probiotic strains should have generally recognized as safe (GRAS) status. To survive passage through the stomach and small intestine, probiotic strains must tolerate the acidic and protease-rich conditions of the stomach, and survive and grow in the presence of bile and acids. Adherent probiotics strains are desirable because they have a greater chance of becoming established in the gastrointestinal tract, thus enhancing their probiotics effect [109]. The production of antimicrobial substances is regarded as important selection criteria for probiotics. Many probiotic *Lactobacillus* species have been shown to produce antimicrobial substances [110]. Ability to modulate immune responses is important for probiotic properties to modulate immune responses in immune dysfunction state. Probiotic microorganisms should also be technologically suitable for incorporation into food products and should be capable of surviving industrial applications [107, 111].

Table 2. Criteria for an ideal probiotic strain [100, 107]

Desirable characteristics of an ideal probiotic microorganisms
Human origin
Generally recognized as safe (GRAS) status
Resistance to gastric acidity and bile toxicity
Adherence to gut epithelial tissue
Ability to colonize the gastrointestinal tract
Production of antimicrobial substances
Ability to modulate immune responses
Amenable to large scale fermentation and commercial production

5.4 Mechanism of action of probiotics

There are many proposed mechanisms by which probiotics may protect the host from intestinal disorders, but the main mechanisms are not fully elucidated. Much work remains to clarify the mechanisms of action of particular probiotics against particular pathogens. In addition, the same probiotic may inhibit different pathogens by different mechanisms [16]. Listed below is a brief description of mechanisms by which probiotics may protect the host against intestinal disease [16, 90].

Production of inhibitory substances

Probiotic bacteria produce a variety of substances that are inhibitory to both gram-positive and gram-negative bacteria. These inhibitory substances include organic acids, hydrogen peroxide, diacetyl, reuterin and bacteriocins as described above. These compounds may reduce not only the number of viable cells but may also affect bacterial metabolism or toxin production [16].

Blocking of adhesion sites

Competitive inhibition for bacterial adhesion sites on intestinal epithelial surfaces is another mechanism of action for probiotics [112-114]. Consequently, some probiotic strains have been chosen for their ability to adhere to epithelial cells. In this way, they may resist peristalsis which would otherwise flush them from the gut. As well as occupying a niche at the expense of potentially harmful organisms, they may specifically block the adherence of enteropathogens [16, 90].

Competition for nutrients

The ability to compete for limiting nutrients is an important factor that determines composition of the gut microbiota, with species that are unable to compete being effectively eliminated from the system. Bacteria in the large intestine are subject to a range of substrate availability; species in the proximal colon have a large supply of nutrients, provided by dietary residues transiting from the small intestine, while those occupying the distal region of the colon have more limited substrate availability. Increasing lactobacilli numbers by way of a probiotic may thereby

decrease the substrate available for other bacterial populations. However, the evidence that this occurs *in vivo* is lacking [16, 90].

Degradation of toxin receptor

The postulated mechanism by which *Saccharomyces boulardii* protects animals against *C. difficile* intestinal disease is through degradation of the toxin receptor on the intestinal mucosa [115, 116].

Stimulation of immunity

Recent evidence suggests that stimulation of specific and nonspecific immunity may be another mechanism by which probiotics can protect against intestinal disease [117].

5.5 Strains used as probiotics

Many microorganisms have been used or considered for use as probiotics [16]. A probiotic preparation may contain one or several different strains of microorganisms. Because viable and biologically active microorganisms are usually required at the target site in the host, it is essential that the probiotics be able to withstand the host's natural barriers against ingested bacteria. The most commonly used probiotics are strains of lactic acid bacteria especially, *Lactobacillus* and *Bifidobacterium* [16]. The beneficial effects of *Lactobacillus* and *Bifidobacterium* have been discussed for decades. Bacteria in these two genera resist gastric acid, bile salts and pancreatic enzymes, adhere to intestinal mucosa and readily colonize the intestinal tract. They are considered important components of the gastrointestinal

microbiota and are relatively harmless. *Lactobacillus* species are typically used as human probiotics because they are easy to cultivate in bulk and have a long history of safe use in fermented foods [118]. As shown in Table 3, *Lactobacillus, Bifidobacterium* and other microorganisms are currently being used as probiotics either singly or in combination. *Lactobacillus* species have been demonstrated to inhibit the *in vitro* growth of many enteric pathogens including *E. coli* O157:H7, *S.* Typhimurium, *Staphylococcus aureus*, *Campylobacter jejuni*, *Clostridium perfringens* and *C.difficile* [36, 119-123]. Therefore, they have been used in both humans and animals to treat a broad range of gastrointestinal disorders [122-123]. Hundreds of publications have described the use of probiotics to prevent and treat a variety of gastrointestinal disorders.

Table 3. Microorganisms considered as probiotics [100]

Lactobacillus species	*Bifidobacterium* species	Others
L. acidophilus	*B. bifidum animalis*	*Bacillus cereus*
L. rhamnosus	*B. longum*	*Clostridium butyricum*
L. gasseri	*B. breve*	*Escherichia coli*
L. casei	"*B. infantis*"	*Proprionibacterium*
L. reuteri	*B. lactis*	*Freundendsreichii*
L. bulgaricus	*B. adolescentis*	"*Saccharomyces boulardii*"
L. plantarum		*Enterococcus feecalis*
L. johnsonii		*Streptococcus thermophilus*
		Lactococcus species
		VSL#3 (*L.bulgaricus, L. plantarum, B. longum, B. infantis, B. breve, S. salivarius* subsp. *thermophilus*)

6. Role of probiotics in intestinal disorders and infectious diarrhea

Antibiotic-induced diarrheal disease

Diarrhea is the most common side effect of antimicrobial therapy with 20% of patients receiving an antibiotic developing this condition [124]. The pathogenesis of antibiotic-induced diarrhea is not understood but is undoubtedly related to quantitative and qualitative changes in the intestinal microbiota [125]. Many of the studies that have attempted to demonstrate the usefulness of probiotics in antibiotic-associated diarrhea have used it prophylactically. However, because of the low incidence of antibiotic-associated diarrhea and the variable intensity of the diarrhea, it is not practical from a cost-benefit viewpoint to treat all patients receiving antibiotic therapy in this way with a probiotic. Furthermore, it is not possible to predict which patient will develop antibiotic-associated diarrhea. Nonetheless, several probiotics have been used in an attempt to prevent antibiotic-associated diarrhea [16]. Adam et al. [34] prospectively treated 388 ambulatory patients, receiving either tetracycline or a β-lactam, concurrently with placebo or "*Saccharomyces boulardii*". The incidence of diarrhea in patients receiving the placebo was 17.5%, whereas in patients receiving "*S. boulardii*", it was 4.5%. These results were confirmed in another study of 193 patients receiving at least one broad-spectrum β-lactam antibiotic [126]. Of the 97 patients receiving *S. boulardii*, only 7.2% developed antibiotic-associated diarrhea compared with 14.6% of the 96 patients receiving placebo. Lactinex, a commercial preparation containing *L .acidophilus* and *L. bulgaricus*, was used in a placebo-controlled study of 79 hospitalized patients receiving ampicillin [127]. The rationale for using *Lactobacillus* in these patients is based on the observation that antibiotic

therapy often causes a loss or reduction in the number of intestinal *Lactobacillus*. Thirty-six patients received concurrent Lactinex and 43 patients received placebo. None of the patients receiving Lactinex developed ampicillin-induced diarrhea, whereas 14% of the placebo group developed diarrhea.

Clostridium difficile-associated intestinal disease

Clostridium difficile is a classic example of the opportunistic proliferation of an intestinal pathogen after breakdown of colonization resistance due to antibiotic administration. After antibiotic intake by animals and humans, *C. difficile* colonizes the intestine and releases two protein exotoxins, toxin A and toxin B, which mediate the diarrhea and colitis caused by this microbe. Toxigenic *C. difficile* is the cause of 20–40% of cases of antibiotic- associated diarrhea [128-130]. The multiple relapses can occur and the relapses can be more severe than the original disease. The mechanism of relapse is unknown but is probably due to the survival of *C. difficile* spores in the intestinal tract until the antibiotic is discontinued [131]. An attractive alternative to antibiotic therapy is to use probiotics to restore intestinal homeostasis. *L. paracasei*, *L. plantarum* and *L. salivarius* have been reported to inhibit several *C. difficile* toxin A-producing strains *in vitro* [121, 30]. In a placebo-controlled study, McFarland et al. [132] examined standard antibiotic therapy with concurrent *S. boulardii* or placebo in 124 adult patients, 64 patients with an initial episode of *C. difficile* disease and 60 patients with a history of at least one prior episode of *C. difficile* disease. The investigators found that in patients with an initial episode of *C. difficile*, there was no significant difference in the recurrence of *C. difficile* disease in the placebo or *S. boulardii* groups. However, in patients with prior *C. difficile* disease, *S. boulardii*

significantly inhibited further recurrences of disease. The investigators concluded that in combination with standard antibiotics, *S. boulardii* is an effective and safe therapy for patients with recurrent *C. difficile* [16].

Rotavirus diarrhea

Rotavirus is a common cause of infantile diarrhea. Rotavirus is a significant cause of infant morbidity and mortality, particularly in developing countries [133, 134]. The principal means of treatment is oral rehydration although an effective vaccine that should decrease dramatically the health impact of rotavirus infections has recently become available. *Lactobacillus* has demonstrated some promise as a treatment for rotavirus infection [135, 136]. Isolauri [137] studied the children with diarrhea treated with either *Lactobacillus* GG or placebo. Approximately 80% of the children with diarrhea were positive for rotavirus. The investigators demonstrated that the duration of diarrhea was significantly shortened in patients receiving *Lactobacillus* GG and the effect was even more significant when only the rotavirus-positive patients were analyzed.

Traveler's diarrhea

The incidence of diarrhea in travelers to foreign countries varies from 20 to 50% depending on the origin and the destination of the traveler, as well as the mode of travel. Although various infectious agents can cause traveler's diarrhea, enterotoxigenic *E. coli* is the most common. Several probiotics have been examined for their ability to prevent traveler's diarrhea, including *Lactobacillus, Bifidobacterium, Streptococcus* and *Saccharomyces* [138-140]. These studies have involved several different groups of travelers such as Finnish travelers to Turkey,

American travelers to Mexico, British soldiers to Belize and European travelers to Egypt. The results from these studies have been extremely variable. For example, in the study of Finnish travelers to Turkey, the travelers had two different destinations [139]. In one destination, *Lactobacillus* GG provided protection against traveler's diarrhea but failed to protect travelers at the other destination. Different etiologic agents may have involved in these two locations, but this possibility was not examined.

Food borne pathogen-associated gastrointestinal infections

Disruption of the normal balance of the resident gastrointestinal microbiota can allow establishment and growth of transient enteropathogens like *Salmonella, Campylobacter, E. coli, Listeria* and *Shigella* spp. Several *Lactobacillus* species have been examined and exhibited antagonistic to *B. cereus, E. coli, S. aureus, Yersinia enterocolitica* and *Listeria in vitro* [120]. In addition, *Lactobacillus* sp., *L. acidophilus, L. plantarum* and *L. brevis* have been shown to inhibit *C. jejuni, E. coli* O157:H7 and *S.* Typhimurium [30, 119]. Studies using animal models have established the ability of certain probiotics to inhibit pathogen growth [90].

***Helicobacter pylori* gastroenteritis**

Helicobacter pylori has been shown to be an important etiologic agent of chronic gastritis as well as gastric and duodenal ulcers. It has also been postulated that chronic *H. pylori* infection leads to stomach carcinoma. *L. acidophilus* and *L. rhamnosus* confer inhibitory effects on *H. pylori in vitro* [141, 142]. With an animal model using gnotobiotic mice, it was found that *L. salivarius* was effective in

inhibiting *H. pylori* [143], while in a human trial, *L. acidophilus* was effective at inhibiting colonization of the organism [144].

7. Role of Probiotics in Inflammatory Bowel Diseases

Inflammatory bowel diseases (IBD) including two forms of Crohn's disease and ulcerative colitis, is a significantly public health in Western societies and their etiologies remain unclear. IBD is characterized clinically by chronic inflammation in the large and/or small intestine. The most common clinical manifestation of ulcerative colitis is an inflammation of the colon. No specific treatment is available for either disease [16]. Evidence suggests that abnormal activation of the mucosal immune system against the enteric microbiota is the key event triggering inflammatory mechanisms that induce mucosal injury and intestinal lesions to chronicity. Patient showed an increased mucosal secretion of IgG antibodies against commensal bacteria [145] and mucosal T-lymphocytes are hyperreactive against antigens of the commensal microbiota, suggesting that local tolerance mechanisms are abrogated [146]. Evidences suggested that TNF-α, quantities in serum, stool and intestinal tissues are elevated in patients with Crohn's disease [147, 40] and imbalance in TNF-α and TNF-α inhibitors plays an important role in gut inflammation in patients with IBD [109]. For instance, TNF-α could induce epithelial cells to secrete IL-8, and express membrane Toll-like receptor 4 (TLR4) excessively [108, 109]. TLR4 could enable intestinal epithelia hyperreactive in response to lipopolysaccharides (LPS), the component of gram-negative bacteria cell walls, and IL-8 has chemotactic and stimulatory properties [42]. As a result, inflammatory cells infiltrate and the inflammatory reaction is therefore increased.

CHAPTER III

MATERIALS AND METHODS

1. Subjects

Newborn infants were recruited from the mothers who delivered at the King Chulalongkorn Memorial Hospital, Bangkok, Thailand. The participant mother answered the questionnaires and gave informed consent. Ethical approval of the present study was obtained from the Ethical Committee of Faculty of Medicine, Chulalongkorn University.

Sixty-five infants were aged 29 to 50 days (mean $\pm$ SD, 35 $\pm$ 5 days), between September 2007 and December 2007. The infants were classified into two groups based on the feeding type. The first group of infants was exclusively breast-fed and the second group was nonexclusively formula-fed (mixed-fed) consisting of infants who received formula-milk and breast-milk. The remaining 2 infants were exclusively formula-fed. They were healthy full-term Thai infants (of both sexes) with no evidence of disease at the first of one month and had no need for antibiotics and all were born by vaginal delivery.

2. Sample collection

Faecal samples were collected at participants' home. About 2-3 g of fresh faeces taken rapidly into two sterile plastic tubes with screw cap. The first tube was cooled in ice box for real-time PCR quantification and the second tube kept at room temperature for bacterial culture. The samples were sent as fresh as possible (within 6 hr) to the laboratory at Faculty of Medicine, Chulalongkorn University for analysis.

3. Quantification of bifidobacteria and lactobacilli by real-time PCR

3.1 DNA extraction from faecal samples

Upon arrival in the laboratory, faecal samples that transport on ice were stored at -80^{0}C directly until analysis. Bacterial DNA was extracted using the QIAamp DNA Stool Kit (Qiagen, Hilden, Germany), with some modifications. Briefly, 0.2 g of frozen faeces were placed in a bead-beating tube filled with 0.3 g of 0.1 mm glass beads, and 1.4 ml of ASL lysis buffer from the stool mini kit was added. The tube was then agitated for 2 min at maximum speed using a Mini Beadbeater-8 (Biospec Products, Bartles-ville, USA). The suspension was incubated at 95^{0}C for 5 min, followed by an additional bead-beating step of 2 min. After centrifugation (5000g, 2 min) to remove cell debris, the supernatant was transferred to a clean vial and an Inhibitex tablet (Qiagen) added to remove DNA-damaging substances and PCR inhibitors. The tablet was dissolved with 3s vigorous agitation, using the bead-beater. DNA was then purified using QIAamp spin columns (Qiagen) as per the manufacturers instructions. The DNA was eluted in a final volume of 200 µl. Finally, DNA samples were stored at -20^{0}C.[48]

3.2 Real- time PCR analysis

PCR primers

The genus-specific 16S rRNA-targeted primers sets used for quantitative real-time PCR in this study are listed in Table 4. *Bifidobacterium* genus-specific primers Bif 164F and Bif 601R have been validated for bifidobacterial specificity by Langendijk[38]and Bernhard [39], respectively. *Lactobacillus* genus-specific primers L159-f and L677-r have been validated for lactobacilli specificity by Heilig et al.[34]

Table 4. Group-specific 16S rRNA-targeted primers used for real-time PCR

Target groups	Primer	Sequence (5'-3')	PCR product Size
Bifidobacteria	Bif 164F	GGG TGG TAA TGC CGG ATG	443 bp
	Bif 601R	TAA GCG ATG GAC TTT CAC ACC	
Lactobacilli	L159-f	GGA AAC AG(A/G) TGC TAA TAC CG	546 bp
	L677-r	CAC CGC TAC ACA TGG AG	

Real- time PCR conditions

PCRs were performed in 20 µl final volumes in capillary tubes in a LightCycler 2.0 instrument (Roche Diagnostic, Mannheim, Germany). Reaction mixtures contained 1X master mix (LightCycler® FastStart DNA Master^{PLUS} SYBR Green I, Roche, Germany), 0.5 µM each primer and 2 µl of respective bacterial template DNA.

All capillaries were sealed, centrifuged at 500g for 5s, and then amplified in a LightCycler instrument. The amplification program for bifidobacterium performing touchdown PCR comprised of activation of polymerase (95°C for 10

min), followed by 40 cycles of 30s at 95°C, 10s at 65°C, 59°C sec target, 0.5°C step size, 1 cycle step delay, and 20s at 72°C. The amplification program for lactobacillus comprised of activation of polymerase (95°C for 3 min), followed by 40 cycles of 40s at 95°C, 25s at 61°C, and 1 min at 72°C. The temperature transition rate was 20°C/s for all steps. Double-stranded PCR product was measured during the 72°C extension step by detection of fluorescence associated with the binding of SYBR Green I to the product. Fluorescence curves were analyzed with LightCycler Software version 4. Melting curves were used to determine the specificity of the PCR. Melting curve analysis was performed immediately after the amplification protocol under the following conditions : 0s (hold time) at 95°C; 15s at 70°C and 0s (hold time) at 95°C. Temperature change rates were 20°C/s, except in the final step, which was 0.1°C/s. After the end of the PCR all products were gradually melted down in 0.1°C increments until all products had been denatured.[48]

The standard curve profiles of bifidobacterium and lactobacillus generated by the LightCycler Software in this study. Quantification of unknowns was achieved by using standard curves made from a standard dilution series of known concentrations of plasmid DNA containing the respective amplicon for each set of primers.[49] The resulting standard curve was shown as a graph of crossing point (Cp) vs. log of known concentration DNA standard. The crossing point was defined as the maximum of the second derivative from the fluorescence curve. All calculated unknown sample values must fall within the limits of the standards used to generate this curve.

3.3 Construct standard curve from plasmid DNA

Amplification target amplicons

For conventional PCR mixture (50 µl) contained 5 µl (10xPCR buffer with 20 mM $MgSO_4$, Promega, Madison, WI, USA), 0.2 mM concentrations of deoxynucleoside triphosphates, 0.5 µM primer, 5 µl of bacterial template DNA, and 2 U of *Pfu* DNA polymerase (Promega). The forward primer Bif164f and reverse primer Bif601r were used to amplify 16S rRNA of the *Bifidobacterium breve* ATCC 15700. A PCR amplification using the following temperature profile : 95^0C for 5 min, followed by 35 cycles comprising of 95^0C for 1 min, 59^0C for 1 min, 72^0C for 1 min, and final extension for 8 min at 72^0C. The forward primer L159F and reverse primer L677R were used to amplify 16S rRNA of the *Lactobacillus plantarum* ATCC 14912. A PCR amplification using the following temperature profile : 95^0C for 5 min, followed by 35 cycles comprising of 95^0C for 30 s, 61^0C for 20 s, 72^0C for 40 s, and final extension for 7 min at 72^0C.

Cloning of PCR products

PCR products were ligated into the pJET1/blunt Cloning Vector (Figure 1), as specified by Fermentas (USA). Competent *E. coli* DH5 cells were transformed with ligation products. LB medium supplemented with ampicillin (200 µg/ml) was used for cloning and subculture. Cells containing the correct plasmid insert were confirmed with conventional PCR and sequencing.

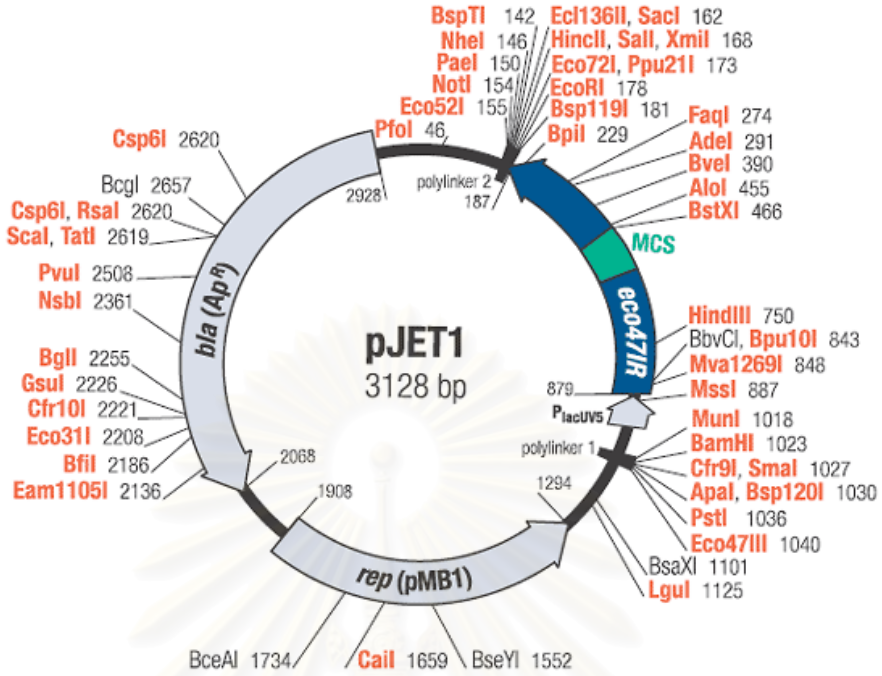


Figure 1. Map and Features of pJET1/blunt Cloning Vector

Plasmid DNA Purification

Plasmid DNA was purified using the NucleoSpin®Plasmid Mini kit. Miniprep concentrations were determined by electrophoresis and comparison of band strengths against molecular marker DNA (1 kb DNA ladder). Linearized plasmid by *Bam*H I Restriction Enzyme (Promega) which cut pJET1/blunt DNA once. Furthermore, the linearized plasmid DNA were purified by QIAquick PCR Purification kit (Qiagen, Hilden, Germany). After spectrophotometric determination of plasmid DNA concentration, the copy number of standard DNA molecules can be calculated according to using the following formula[45] :

(Xg/µl DNA / [plasmid length in basepairs x 660]) x 6.022 x 10^{23} = Y molecules/ µl

3.4 Evaluation of the intra- and inter-assay variation

Plasmid standards and samples were simultaneously assayed in duplicate and for two independent experiments. Plasmid DNA concentrations 10^2, 10^4, 10^6 and 10^8 copies were used in each real-time PCR assay (Figure 2).

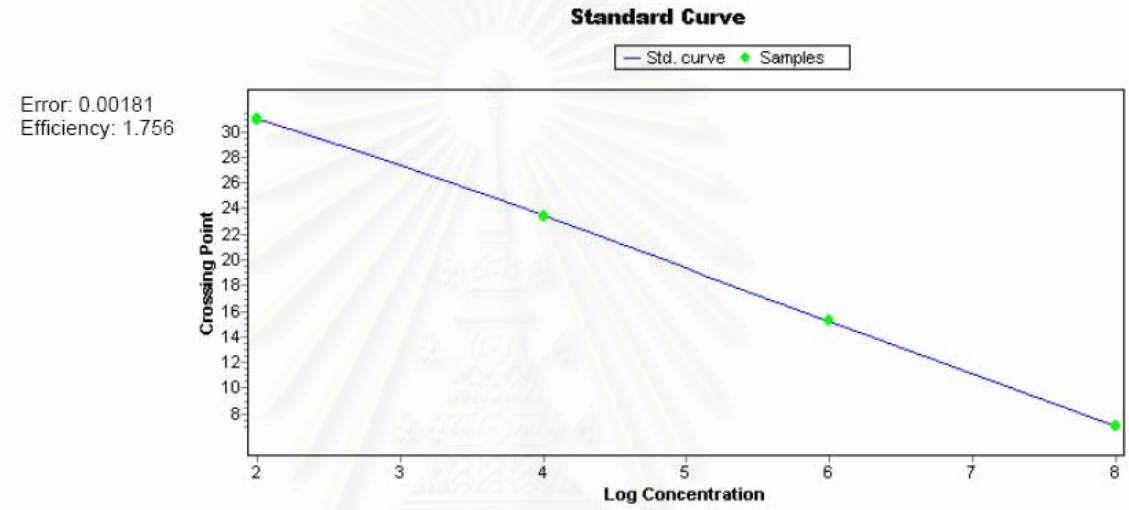


Figure 2. Standard curve generated by plasmid DNA concentrations 10^2, 10^4, 10^6 and 10^8 in duplicate for quantitation of target DNA in test sanples.

3.5 Data analysis

For each assay results obtained by PCR were converted to the bacterial 16S rRNA genes per gram wet weight faeces. Conversion of bacterial 16S rRNA genes to the number of bacterial cells in a sample was omitted, with varying ribosomal DNA copy numbers of each target bacteria group were while differences in the rrn copy numbers.

Copy numbers of 16S rRNA genes of bifidobacteria or lactobacilli per gram of sample were transformed into logarithms and normal distributed data were subjected to statistical analysis. Student's *t*-test was used for comparison of means. All tests were two-tailed, and *p* values <0.05 were accepted as statistically significant.

4. Isolation of bifidobacteria and lactobacilli from fecal samples

Upon arrival in the laboratory, fecal samples that were transported at room temperature were processed immediately for bacterial isolation. Approximately 1 g of the specimen was weighed and suspended in 9 ml (10% [wt/vol]) pre-reduced buffered peptone water[43,44,45] and ten-fold serial diluted to 10^{-2}-10^{-7} in reduced physiological salt solution (RPS).[44,45] Finally, 100 µl of 10^{-4},10^{-5},10^{-6},10^{-7} diluted samples were spreaded onto modified Columbia medium (MC) containing 0.03 g/l bromocresol purple[46,47] to isolate bifidobacteria and deMan-Rogosa-Sharpe (MRS) agar plates (Oxoid Ltd, Basingstoke, Hampshire, UK) to isolate lactobacilli. The plates were incubated under anaerobic condition at 37°C for 48-72 hr in an anaerobic chamber (the AnaeroPack system, Mitsubishi Gas Chemical, H:5%, CO2:10%, N:85%).

All colonies that developed on both MC (yellow zones) and MRS agar with different morphologies were selected. Four-seven colonies of each bacterial morphotype were randomly picked, separately Gram-stained, microscopically examined, catalase tested and subcultured to purify on the same media. One representative isolate from each colony phenotype was further analysed. All Gram-positive bifid-shaped rods were tentatively considered *Bifidobacterium*-like bacteria.

All Gram-positive, non-spore forming straight rods or short rods were tentatively considered *Lactobacillus*-like bacteria. Bifidobacteria and lactobacilli isolates were identified by molecular methods. Pure isolate of bifidobacteria were maintained in 20% glycerol brain heart infusion broth (BHB) and pure isolate of lactobacilli were maintained in 20% glycerol MRS broth as frozen cultures at -80°C for experimental use.

5. Antagonistic Activity Assay by Using Agar Spot Method

The agar spot method was carried out as previously described [49] with modification by Splinler et al.[48] This method used to detect antimicrobial activities of *Bifidobacterium* and *Lactobacillus* against various bacterial strains. The following target bacteria used in the present investigation were obtained from the culture collection of the Department of Medical Science, Ministry of Public Health, Thailand. These seven gastrointestinal pathogens were enterotoxigenic *E. coli* (ETEC) DMST 20970, enteroinvasive *E. coli* (EIEC) DMST 20971, enteropathogenic *E. coli* (EPEC) DMST 20972, enterohemorrhagic *E. coli* (EHEC) DMST 20973, *Salmonella* Typhimurium ATCC 13311, *Shigella flexneri* DMST 4423 and *Vibrio cholerae* non O1 DMST 2873. All target bacteria were grown on 5% Sheep blood agar and subculture on tryptic soy broth under aerobic condition at 37°C, 24 hr for experimental use.

5.1 Anti-microbial activity of bifidobacteria

The frozen cultures of *Bifidobacterium* were precultivated on MC agar for 48-72 hr in an anaerobic environment and subcultured on BHI broth in a 96 well plate. Forty eight hour cultures of *Bifidobacterium* isolates in each well of 96-well plate were spotted by using a frogger (DAN-KAR CCRP, MA, USA) onto the surface of BHI agar in 140 mm large plate. The spots were developed after incubation in an anaerobic condition at 37°C for 48-72 hr. The *Bifidobacterium* spots in each plate were overlaid with 20 ml of tryptic soft agar (agar 7.5 g/l) and each of overnight culture of pathogen at a final concentration of 1×10^7 cells/ml. Plates were allowed to solidify for 5 min and then the plates were incubated aerobically at 37°C for 24 hr. Inhibition zones were measured after 18-24 hr and a clear zone of equal or more than 1 mm around a spot was scored as positive. These assays were performed three times in triplicate.

5.2 Anti-microbial activity of lactobacilli

The frozen cultures of *Lactobacillus* were precultivated on MRS agar for 48 hr in an anaerobic environment, a single colony was isolated and subcultured on MRS broth two times in 96 well plate. Twenty four hour cultures of *Lactobacillus* isolates in each well of 96-well plate were spotted by using a frogger onto the surface of BHI agar supplemented with 20 mM glucose in 140 mm large plate. The spots were developed after incubation in an anaerobic condition at 37°C for 24 hr. The *Lactobacillus* spots in each plate were overlaid with 20 ml of tryptic soft agar (agar

7.5 g/l) and each of overnight culture of pathogen at a final concentration of 1 x 10^7 cells/ml. Plates were allowed to solidify for 5 min and then the plates were incubated aerobically at 37°C for 24 hr. Inhibition zones were measured after 18-24 hr and a clear zone of equal or more than 1 mm around a spot was scored as positive. These assays were performed three times in triplicate.

6. Identification of bifidobacteria and lactobacilli at the species level

6.1 DNA extraction

For DNA extraction, the frozen cultures were thawed on ice. Cells were harvested at 4°C and washed with water. Cell pellets were resuspended in 1X digestion buffer (stock 10X digestion buffer contained 5%tween20 and 10 mg/ml proteinase K in 0.2 M Tris pH 8.3) and incubate 60°C, 1 h inactivated proteinase K at 100°C 15 min centrifugation at 13,000 rpm 5 min used DNA supernatant to PCR reaction.

6.2 PCR amplification

PCR primers

Internal-transcribed spacer polymerase chain reaction (ITS-PCR) was used to confirm the species identification of bifidobacteria as demonstrated in previous studies using primers Bifido-16S-1f and Bifido-ITS-1r.[23] Genotypic characteristic of lactobacilli by 16S rRNA gene sequencing was performed according

to the method as previously described using primers primers 16S-8F and 16S-1541R [5]. The primers set used for DNA sequencing in this study are listed in Table 5.

Table 5. Primers used for DNA sequencing

Target groups	Primer	Sequence (5'-3')	PCR product Size
Bifidobacteria	Bifido-16S-1f	TCC AGG GCT TCA CGC ATG C	600 bp
	Bifido-ITS-1r	TCC AGT TCT CAA ACC ACC AC	
Lactobacilli	16S-8F	AGA GTT TGA TCY TGG YTY AG AAG	1,550 bp
	16S-1541R	AAG GAG GTG WTC CAR CC	

PCR conditions

PCR was performed in a programmable thermal cycler. The typical reaction mixture (50 ul) for PCR of the 16S and ITS regions consisting of 1X reaction buffer, 2mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 pM of each primer, 5 µl of bacterial template DNA, and 2U of FastStart Taq DNA polymerase (Roche, Germany). Each reaction was carried out for 5 min at 95°C; 35 cycles of 1 min at 95°C, 1 min at 60°C, 2 min at 72°C, and 8 min at 72°C. The template DNA of lactobacillus 16S rRNA gene was amplified in a total volume of 50 µl containing of 1X reaction buffer, 3 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 µM of each primer, 2 µl of bacterial template DNA, and 1.25U of FastStart Taq DNA polymerase (Roche, Germany).Amplification program was performed : 95°C for 5 min; 35 cycles of 95°C for 30 s, 57°C for 1 min, and 72°C for 1 min and a final extension of 72°C for 5 min. The PCR products were electrophoresed in 2% agarose gel that were stained by ethidium bromide and photographed under UV-light

The PCR products were purified before sequencing using QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). Sequencing was carried out by 1st BASE ( ) Sequencing. A search of the GenBank DNA database was conducted by using the BLAST algorithm (http://www.ncbi.nlm.gov/BLAST). In general, when similarity values exceed 98%, the strains were considered to belong to the same species.

CHAPTER IV

RESULTS

1. Population

Sixty- five faecal samples were collected from 30 exclusively breast-fed infants, 2 exclusively formula-fed infants and 33 mixed-fed infants. Therefore, the 2 exclusively formula-fed infants were withdrawn from this study and the infants were classified into two groups based on the feeding method, exclusively breast-fed infants (n = 30) and mixed-fed infants (n = 33). Subjects and feeding method were shown in Table 6. and formula milk component was shown in appendix E.

Table 6. Sixty- five infants participants and feeding method

No.	Subjects No.	Sexes	Feeding type	Age (days)
1	12	M	Breast milk	29
2	13	F	Breast milk	30
3	14	F	Breast milk and formula milk	29
4	15	M	Breast milk	29
5	16	F	Breast milk and formula milk	32
6	17	M	Breast milk	33
7	18	M	Breast milk	34
8	19	F	Breast milk	30

M = Male F = Female

Table 6. Sixty five infants participants and feeding method. (continued)

No.	Subjects No.	Sexes	Feeding type	Age (days)
9	20	M	Breast milk	30
10	21	M	Breast milk and formula milk	30
11	22	M	Breast milk and formula milk	31
12	23	F	Breast milk and formula milk	30
13	24	M	Breast milk, formula milk and banana	32
14	25	M	Breast milk	35
15	26	M	Breast milk and formula milk	33
16	27	M	Breast milk and formula milk	34
17	28	M	Breast milk and formula milk	32
18	29	M	Breast milk, formula milk and banana	34
19	30	F	Breast milk and formula milk	30
20	31	F	Breast milk and formula milk	29
21	32	M	Breast milk and formula milk	31
22	33	M	Breast milk and formula milk	29
23	34	F	Formula milk	30
24	35	F	Breast milk	32
25	36	M	Breast milk and formula milk	32
26	37	F	Breast milk and formula milk	32
27	38	M	Breast milk	33
28	39	M	Breast milk	36
29	40	M	Breast milk and formula milk	32

M = Male F = Female

Table 6. Sixty five infants participants and feeding method. (continued)

No.	Subjects No.	Sexes	Feeding type	Age (days)
30	41	M	Breast milk	33
31	42	F	Breast milk	33
32	43	F	formula milk	34
33	44	F	Breast milk and formula milk	34
34	45	F	Breast milk	36
35	46	F	Breast milk and formula milk	37
36	47	F	Breast milk and formula milk	38
37	48	F	Breast milk and formula milk	39
38	49	M	Breast milk	41
39	50	F	Breast milk and formula milk	43
40	51	F	Breast milk	37
41	52	F	Breast milk	38
42	53	F	Breast milk	37
43	54	F	Breast milk	40
44	55	F	Breast milk	41
45	56	M	Breast milk	45
46	57	F	Breast milk, formula milk and banana	34
47	58	F	Breast milk	44
48	59	M	Breast milk	35
49	60	M	Breast milk and formula milk	37
50	61	F	Breast milk and formula milk	37

M = Male F = Female

Table 6. Sixty five infants participants and feeding method. (continued)

No.	Subjects No.	Sexes	Feeding type	Age (days)
51	62	M	Breast milk and formula milk	37
52	63	F	Breast milk	50
53	64	F	Breast milk	39
54	65	M	Breast milk and formula milk	36
55	66	F	Breast milk	35
56	67	M	Breast milk and formula milk	39
57	68	M	Breast milk and formula milk	43
58	69	F	Breast milk	40
59	70	M	Breast milk and formula milk	35
60	71	M	Breast milk	38
61	72	M	Breast milk and formula milk	37
62	73	M	Breast milk and formula milk	39
63	74	M	Breast milk	38
64	75	F	Breast milk and banana	42
65	76	F	Breast milk and formula milk	48

M = Male F = Female

2. Quantification of bifidobacteria and lactobacilli in infants faeces

Quantification of bifidobacteria and lactobacilli in faeces of 30 breast-fed infants and 33 mixed-fed infants were determined by real time PCR as shown in table 7 and figure 3. The number of bifidobacteria appeared slightly higher in mixed-fed infants than in breast-fed infants (mean values 8.42 and 8.26 $\log_{10}$ 16S rRNA genes/g wet weight faeces, respectively; $p = 0.67$), and the number of lactobacilli appeared slightly higher in mixed-fed infants than in breast-fed infants (mean values 7.20 and 6.84 $\log_{10}$16S rRNA genes/g wet weight faeces, respectively; $p = 0.14$). There were no significant differences in the number of bifidobacteria and lactobacilli between the different feeding groups.

DNA melting curves were used to monitor product specificities. The specific *Bifidobacterium* product melted at 91 $\pm$ 0.5^0C and the specific *Lactobacillus* product melted at 88 $\pm$ 0.5^0C. The sensitivity of these assays could detect as low as 10 copies of specific bacterial 16S rRNA gene as shown in Figure 2.

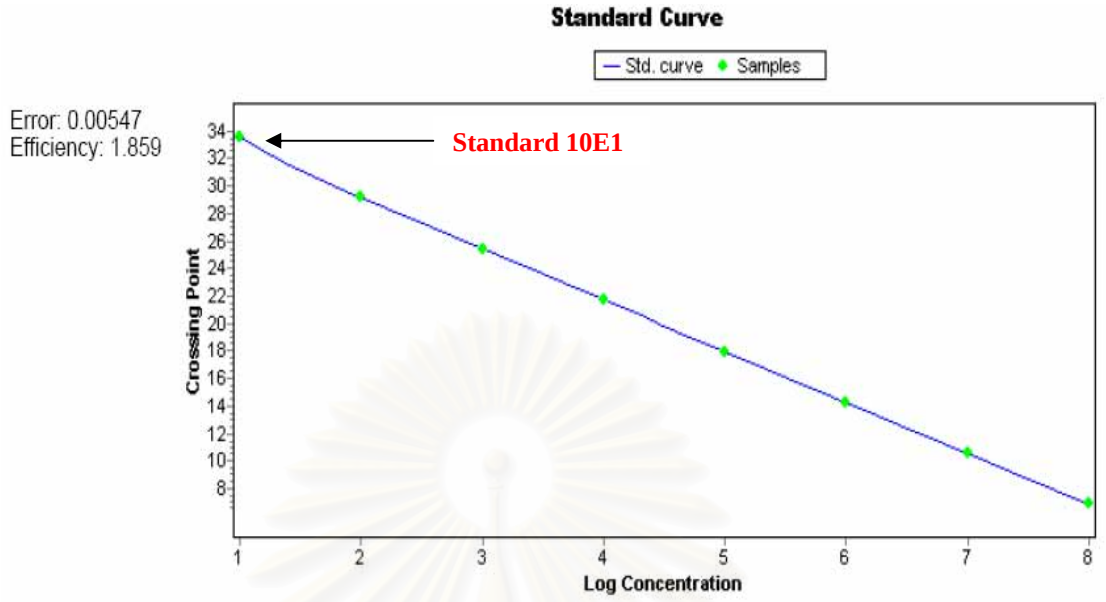


Figure 2. Standard curve of plasmid DNA concentrations of *Lactobacillus* amplicon are plotted against the crossing point. In this figure a sample with an initial DNA only 10 units has a crossing point of 34 amplification cycles.

Table 7. Quantification of bifidobacteria and lactobacilli in faeces of breast-fed (BF) and mixed-fed (MF) infants

Organism	Log_{10} 16S rRNA genes /g wet weight faeces		
	Breast-fed (n = 30)	Mixed-fed (n = 33)	*p*-value[a]
	Mean ± SD	Mean ± SD	
Bifidobacteria	8.26 ± 1.42	8.42 ± 1.47	0.67
Lactobacilli	6.84 ± 0.83	7.20 ± 1.02	0.14

[a] Determined by the independent sample *t*-test. Significant difference $P < 0.05$.

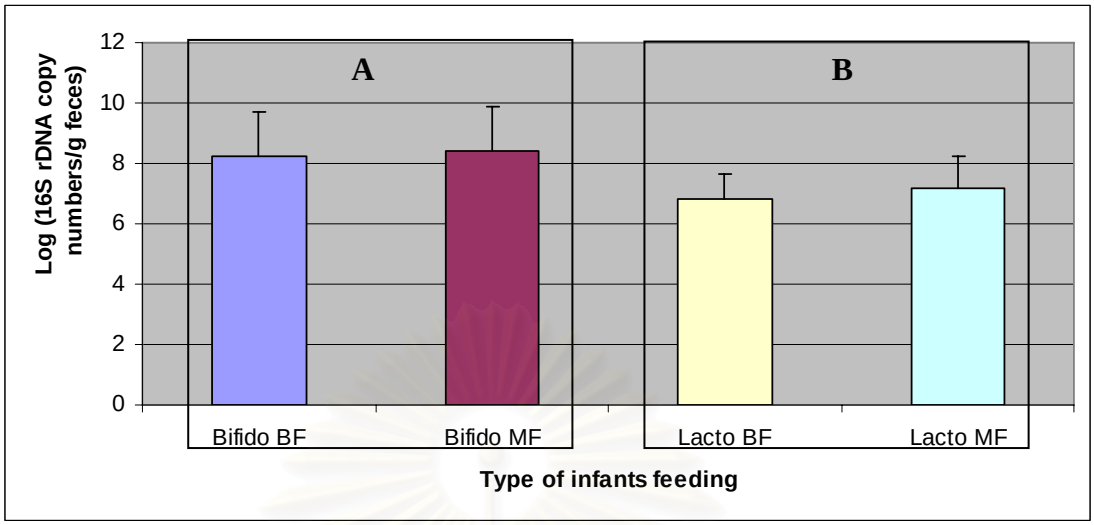
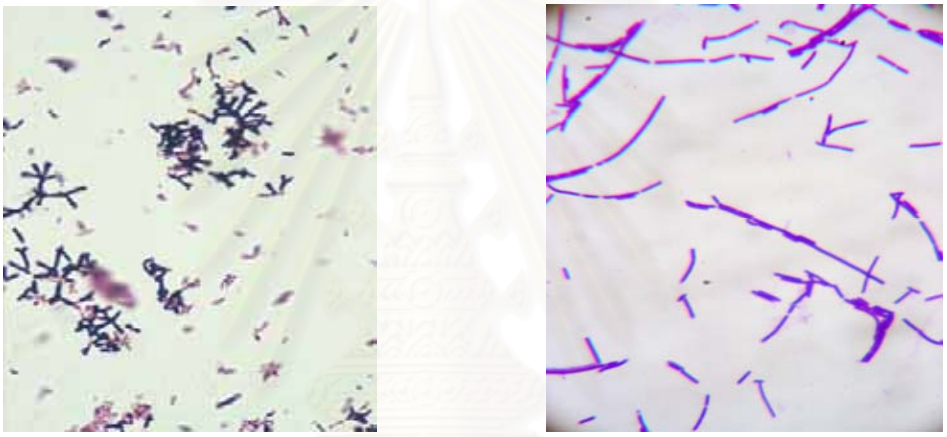


Figure 3. Quantification of bifidobacteria (A) and lactobacilli (B) in faeces of breast-fed (BF) and mixed-fed (MF) infants.

3. Isolation of bifidobacteria and lactobacilli from faecal samples

Bacteria were isolated from 65 healthy infants participants and selected for genus *Bifidobacterium* and *Lactobacillus* by presumptive tests including Gram stain, cell morphology and catalase test. Five hundred and seven isolates were obtained, 391 isolates were suspected to be *Bifidobacterium* and 116 isolates were suspected to be *Lactobacillus*. They were all gram-positive, catalase-negative. *Bifidobacterium* cell morphology in each isolate varied from short, regular, thin cells with pointed ends, coccoidal regular cells, long cells with slight bends or protuberances or with a large variety of branchings; pointed, slightly bifurcated club-shaped or spatulated extremities; single or in chains of many elements; in star-like aggregates or disposed in “V” or “palisade” arrangements. Colonies smooth, convex, entire edges, cream to white, glistening and of soft consistency. *Lactobacillus* cell morphology in each

isolate varied from long and slender rods, straight rods to bent rods, sometimes shot rods to coccobacilli; arranged in single, in pairs, or short chain formation. Some isolates exhibited bipolar staining or internal granulations. The most frequently found colonies varied from small to medium colonies (1-2 mm) with white, circular, smooth and convex colonial morphologies. Most isolates grew well under anaerobic conditions. (see Figure 4)



A. **B**.

Figure 4. **A.** Cell morphology of *Bifidobacterium*

B. Cell morphology of *Lactobacillus*.

4. Antagonistic Activity of *Bifidobacterium* and *Lactobacillus* Isolates Against Gastrointestinal Pathogens by Agar Spot Method

One hundred and eleven isolates of *Bifidobacterium*-like organism and 39 isolates of *Lactobacillus*-like organism that were representative isolate of each individual infant were selected for antagonistic activity assay. The character of inhibitory activity have a clear zone (C) and microcolonies (M) to reveal that the test organisms inhibited all of the pathogen indicator strain and the test organisms inhibited some of the pathogen populations as demonstrated in Figure 5,6. Strains which showed inhibitory activity were repeated by spotted (2 μl) separately onto surface of media. Figure 7,8. A clear zone of inhibition revealed that weak inhibitory activity (1-2 mm) to medium inhibitory activity (3-4 mm). One hundred and six from 111 *Bifidobacterium* tested organisms were observed the inhibitory activity as demonstrated in Table 8. Fourteen from 39 *Lactobacillus* tested organisms were observed the inhibitory activity as demonstrated in Table 9. The results demonstrated that almost *Bifidobacterium* have an inhibitory activity against *Vibrio cholerae* and *Shigella flexneri* and have partial inhibition against ETEC, EIEC, EPEC, EHEC and *Salmonella* Typhimurium. The positive results of the tested *Lactobacillus* have only weak inhibitory activities against *V. cholerae* and some isolated have partial inhibition against *S.* Typhimurium, *Sh. flexneri* and *V. cholerae*.

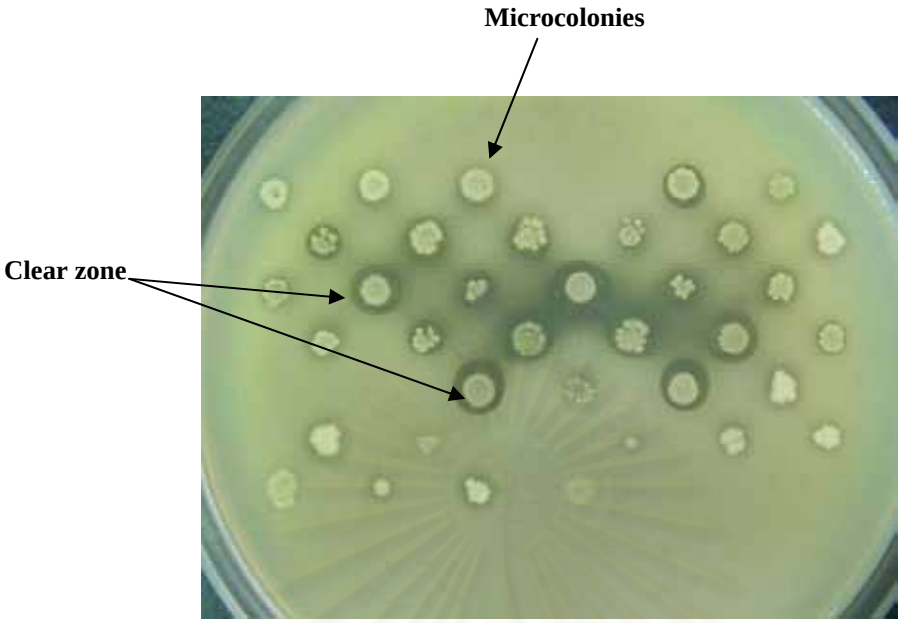


Figure 5. Tested bifidobacteria grown on BHI agar (150 mm plate) anaerobically at 37^0C , 48 hr overlain with 10^7 CFU/ml *Shigella flexneri* and incubated at 37^0C in aerobic condition for 24 hr.

Figure 6. Tested lactobacıiıı grown on ∠0 mM glucose BHI agar (150 mm plate) anaerobically at 37^0C for 48 hr, overlain with 10^7 CFU/ml *Vibrio cholerae* and incubated at 37^0C in aerobic condition for 24 hr.

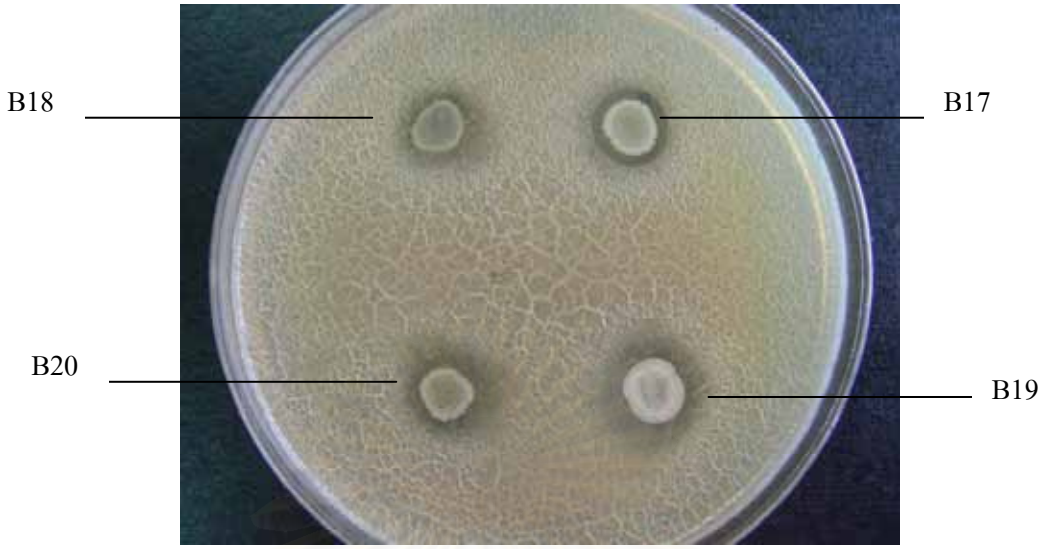


Figure 7. Bifidobacteria spots (2µl) grown separately on BHI agar anaerobically at 37^0C , 48-72 hr overlain with 10^7 CFU/ml *Vibrio cholerae* and incubated at 37^0C in aerobic condition for 24 hr.

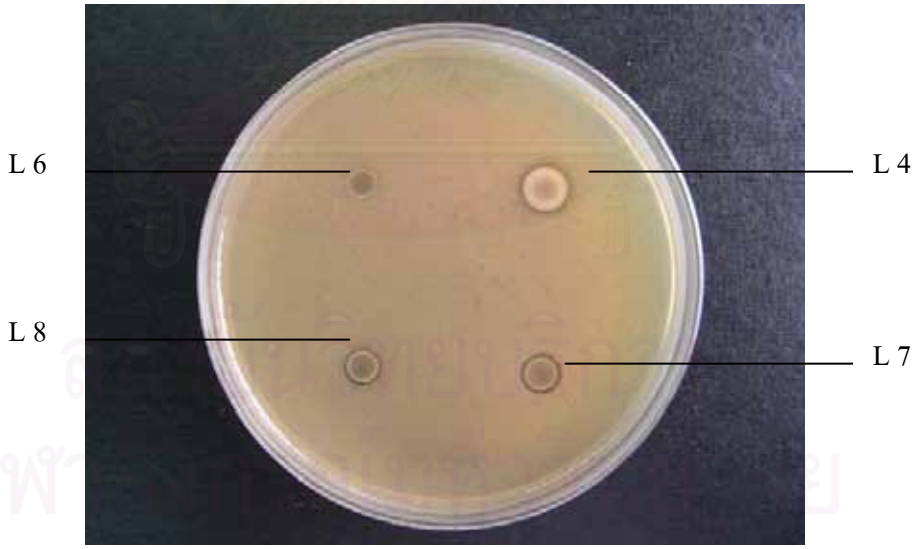


Figure 8. Lactobacilli spots (2µl) grown separately on 20 mM glucose BHI agar anaerobically at 37^0C, 48 hr overlain with 10^7 CFU/ml *Vibrio cholerae* and incubated at 37^0C in aerobic condition for 24 hr.

Table 8. Antagonistic activity of bifidobacteria isolated from infant faeces.

Bifidobacterium isolates	Inhibition of indicator microorganisms						
	ETEC	**EIEC**	**EPEC**	**EHEC**	***Salmonella* Typhimurium**	***Shigella flexneri***	***Vibrio cholerae***
B 1	M	M	M	M	M	M	M
B 2	M	M	M	-	M	M	M
B 3	M	M	M	M	M	M	M
B 4	-	-	-	-	M	M	M
B 5	M	M	M	M	M	C(+)	C(++)
B 6	M	M	M	-	-	M	M
B 7	M	M	M	M	M	M	M
B 8	M	M	M	M	M	C(+)	C(+)
B 9	M	M	M	M	M	C(+)	M
B 10	M	M	M	M	M	M	M
B 11	M	M	M	M	M	M	M
B 12	M	M	M	M	M	M	C(+)
B 13	M	M	M	M	M	M	M
B 14	M	M	M	-	M	C(+)	C(+)
B 15	M	M	M	M	M	C(+)	C(++)
B 16	M	M	M	-	M	C(+)	C(+)
B 17	M	M	M	M	M	C(+)	C(++)
B 18	M	M	M	M	M	C(+)	C(+)
B 19	M	M	M	M	M	M	C(+)

- : No inhibition zone , C : Clear zone (zone size) ; + = 1-2 mm ++ = 3-4 mm , M : Microcolonies ; the indicator strain grew as microcolonies around *Bifidobacterium* spot (partial inhibition)

Table 8. Antagonistic activity of bifidobacteria isolated from infant faeces. (Cont.)

Bifidobacterium **isolates**	**Inhibition of indicator microorganisms**						
	ETEC	**EIEC**	**EPEC**	**EHEC**	***Salmonella*** **Typhimurium**	***Shigella flexneri***	***Vibrio cholerae***
B 20	M	M	M	M	M	M	M
B 21	M	M	M	M	M	C(+)	C(+)
B 22	M	M	M	M	M	C(+)	C(+)
B 23	M	M	M	M	M	M	C(+)
B 24	M	M	M	M	M	C(+)	C(+)
B 25	M	M	M	M	M	C(+)	C(+)
B 26	M	M	M	M	M	C(+)	C(+)
B 27	M	M	M	M	M	C(+)	C(+)
B 30	M	M	M	M	M	C(+)	M
B 31	M	M	M	M	M	C(++)	C(++)
B 32	M	M	M	-	M	C(+)	C(+)
B 35	M	M	M	M	M	C(+)	C(+)
B 36	M	M	M	M	M	M	M
B 37	M	M	M	M	M	M	M
B 38	M	M	M	M	M	C(+)	C(+)
B 40	M	M	M	M	M	C(+)	C(+)
B 41	M	M	M	M	M	C(+)	C(+)
B 44	M	M	M	M	-	M	M
B 46	M	M	M	M	-	M	M

- : No inhibition zone , C : Clear zone (zone size) ; + = 1-2 mm ++ = 3-4 mm , M : Microcolonies ; the indicator strain grew as microcolonies around *Bifidobacterium* spot (partial inhibition)

Table 8. Antagonistic activity of bifidobacteria isolated from infant faeces. (Cont.)

Bifidobacterium isolates	Inhibition of indicator microorganisms						
	ETEC	**EIEC**	**EPEC**	**EHEC**	***Salmonella* Typhimurium**	***Shigella flexneri***	***Vibrio cholerae***
B 48	-	M	M	-	-	M	M
B 49	M	M	M	M	M	M	M
B 50	M	M	M	M	M	M	M
B 51	M	M	M	M	M	M	M
B 52	-	-	-	-	-	M	M
B 53	M	M	M	M	M	C(+)	C(+)
B 54	-	-	-	-	M	C(+)	C(+)
B 56	M	M	-	M	M	M	M
B 57	M	M	M	M	M	C(+)	C(+)
B 58	M	M	M	M	M	M	M
B 59	M	M	M	M	M	C(+)	C(+)
B 60	-	M	M	M	M	C(+)	C(+)
B 61	-	M	M	M	M	C(+)	C(++)
B 63	M	M	M	M	M	C(++)	C(++)
B 66	-	M	M	-	M	C(+)	M
B 69	-	-	-	-	-	M	M
B 70	M	M	M	M	M	M	M
B 72	M	M	M	M	M	C(+)	C(+)
B 73	-	M	M	-	M	M	M

- : No inhibition zone , C : Clear zone (zone size) ; + = 1-2 mm ++ = 3-4 mm , M : Microcolonies ; the indicator strain grew as microcolonies around Bifidobacterium spot (partial inhibition)

Table 8. Antagonistic activity of bifidobacteria isolated from infant faeces. (Cont.)

Bifidobacterium isolates	Inhibition of indicator microorganisms						
	ETEC	**EIEC**	**EPEC**	**EHEC**	***Salmonella* Typhimurium**	***Shigella flexneri***	***Vibrio cholerae***
B 74	-	M	M	M	M	M	M
B 75	M	M	M	M	M	C(+)	M
B 76	M	M	M	M	M	C(+)	C(++)
B 77	M	M	M	M	M	C(+)	C(++)
B 79	M	M	M	M	M	C(+)	C(+)
B 82	M	M	M	M	M	C(+)	C(+)
B 85	M	M	M	M	M	C(+)	M
B 86	M	M	M	M	M	C(+)	M
B 87	M	M	M	-	M	M	M
B 89	M	M	M	M	M	M	M
B 90	M	M	M	M	M	M	M
B 91	M	M	M	M	M	C(+)	C(+)
B 92	M	M	M	M	M	C(+)	C(+)
B 93	M	M	M	M	M	C(+)	C(+)
B 94	M	M	M	M	M	C(+)	C(+)
B 97	M	M	M	M	M	C(+)	C(+)
B 98	-	M	M	M	M	C(+)	C(+)
B 99	M	M	M	M	M	C(+)	C(+)
B 100	-	M	M	-	M	M	M

- : No inhibition zone , C : Clear zone (zone size) ; + = 1-2 mm ++ = 3-4 mm , M : Microcolonies ; the indicator strain grew as microcolonies around *Bifidobacterium* spot (partial inhibition)

Table 8. Antagonistic activity of bifidobacteria isolated from infants faeces. (Cont.)

Bifidobacterium **isolates**	**Inhibition of indicator microorganisms**						
	ETEC	**EIEC**	**EPEC**	**EHEC**	***Salmonella*** **Typhimurium**	***Shigella flexneri***	***Vibrio cholerae***
B 101	M	M	M	M	M	C(+)	C(+)
B 102	-	M	M	-	M	C(+)	C(+)
B 103	-	M	M	-	M	M	C(+)
B 104	-	M	-	-	-	M	M
B 105	-	M	M	M	M	M	M
B 106	-	M	M	M	M	M	M
B 107	M	M	M	M	M	M	M
B 108	M	M	M	M	M	C(+)	M
B 109	M	M	M	M	M	C(+)	M
B 110	M	M	M	M	M	M	M
B 111	M	M	M	M	M	M	M
B 112	-	M	-	-	-	M	M
B 113	M	M	M	M	M	M	M
B 114	M	M	M	M	M	C(+)	C(+)
B 115	-	M	M	M	M	M	M
B 116	-	M	M	M	M	M	M
B 119	-	M	M	M	M	M	M
B 120	-	M	-	M	M	M	M
B 121	-	M	M	M	M	M	M

- : No inhibition zone , C : Clear zone (zone size) ; + = 1-2 mm ++ = 3-4 mm , M : Microcolonies ; the indicator strain grew as microcolonies around *Bifidobacterium* spot (partial inhibition)

Table 8. Antagonistic activity of bifidobacteria isolated from infant faeces. (Cont.)

Bifidobacterium **isolates**	**Inhibition of indicator microorganisms**						
	ETEC	**EIEC**	**EPEC**	**EHEC**	***Salmonella*** **Typhimurium**	***Shigella flexneri***	***Vibrio cholerae***
B 122	M	M	M	M	M	C(+)	C(+)
B 124	-	M	M	M	M	M	M
B 125	-	M	M	M	M	M	M
B 126	-	M	M	-	M	M	M
B 127	-	M	-	M	-	M	M
B 128	-	M	M	M	-	M	M
B 129	-	M	M	-	-	M	M
B 130	-	M	M	M	-	M	M
B 131	-	M	M	M	M	M	M
B 132	M	M	M	M	M	C(+)	C(++)
B 133	M	M	M	M	M	C(+)	C(++)

- : No inhibition zone , C : Clear zone (zone size) ; + = 1-2 mm ++ = 3-4 mm , M : Microcolonies ; the indicator strain grew as microcolonies around *Bifidobacterium* spot (partial inhibition)

Table 9. Antagonistic activity of lactobacilli isolated from infant faeces.

Lactobacillus **isolates**	**Inhibition of indicator microorganisms**						
	ETEC	**EIEC**	**EPEC**	**EHEC**	***Salmonella*** **Typhimurium**	***Shigella flexneri***	***Vibrio cholerae***
L 4	-	-	-	-	-	M	M
L 6	-	-	-	-	-	M	C(+)
L 7	-	-	-	-	-	-	C(+)
L 8	-	-	-	-	-	M	C(+)
L 9	-	-	-	-	-	-	C(+)
L 12	-	-	-	-	-	-	C(+)
L 17	-	-	-	-	-	-	C(+)
L 18	-	-	-	-	-	-	C(+)
L 31	-	-	-	-	M	M	M
L 33	-	-	-	-	M	M	M
L 34	-	-	-	-	M	M	M
L 35	-	-	-	-	M	M	M
L 36	M	M	M	M	M	M	M
L 39	-	-	-	-	M	M	M

- : No inhibition zone , C : Clear zone (zone size) ; + = 1-2 mm ++ = 3-4 mm , M : Microcolonies ; the indicator strain grew as microcolonies around *Lactobacillus* spot (partial inhibition)

5. Genotypic identification of the isolates at species level

One hundred and eleven isolates of *Bifidobacterium* and 39 isolates of *Lactobacillus* that selected to antagonistic activity assay were characterized by DNA sequencing. The genomic DNA of these strains were extracted.

Bifidobacteria 16S rRNA and internal transcribed spacer (ITS) regions were amplified using the primers Bifido-16S-1f and Bifido-ITS-1r (PCR product 600 bp) and *Lactobacillus* 16S rRNA genes were amplified using the primers 16S-8F and 16S-1541R (PCR product 1,550 bp). Purified PCR products were sequenced with the same forward and reverse primer. The bases sequences displayed as N at the beginning and terminal of sequences were excluded and then analyzed by using the sequence match program at the BLAST database search program (http://www.ncbi.nlm.nih.gov/BLAST). The highest similarity value closely related to 100% was used for species identification. The sequence of 16S rRNA genes and 90-100% closet match organism of each *Bifidobacterium* and *Lactobacillus* was displayed in Tables 10-11. These tables demonstrated identity of closet match organism.

Genotypic identification demonstrated that the *Bifidobacterium* and *Lactobacillus* species in faeces of Thai infants at the age of 1 month included *B. longum, B. bifidum, B. adolescentis, B. pseudocatenulatum* and *L. gasseri, L. salivarius, L. fermentum, L. ruminis, L. mucosae, L. vaginalis, L. oris, L. rhamnosus* and *L. casei,* respectively.

Table 10. Genotypic identification of *Bifidobacterium* based on 16S rRNA and ITS regions sequencing.

Subjects No.	***Bifidobacterium* isolates**	**Match organism**	**% Identity**
12	B 1	*Bifidobacterium longum*	100
	B 2	*Bifidobacterium longum*	100
	B 3	*Bifidobacterium longum*	100
14	B 4	*Bifidobacterium bifidum*	100
15	B 5	*Bifidobacterium adolescentis*	90
	B 6	*Bifidobacterium longum* subsp. infantis	91
	B 7	*Bifidobacterium longum* subsp. infantis	92
16	B 8	*Bifidobacterium adolescentis*	91
17	B 9	*Bifidobacterium longum*	99
	B 10	*Bifidobacterium longum*	100
18	B 11	*Bifidobacterium pseudocatenulatum*	99
21	B 12	*Bifidobacterium adolescentis*	91
22	B 13	*Bifidobacterium longum*	90
	B 14	*Bifidobacterium adolescentis*	98
23	B 15	*Bifidobacterium adolescentis*	90
	B 16	*Bifidobacterium adolescentis*	98
	B 17	*Bifidobacterium adolescentis*	90
	B 18	*Bifidobacterium adolescentis*	98

Table 10. Genotypic identification of *Bifidobacterium* based on 16S rRNA and ITS regions sequencing. (Continued)

Subjects No.	*Bifidobacterium* isolates	Match organism	% Identity
24	B 19	No PCR product	
	B 20	*Bifidobacterium adolescentis*	98
	B 21	*Bifidobacterium adolescentis*	98
	B 22	*Bifidobacterium longum*	100
26	B 23	*Bifidobacterium longum*	100
	B 24	*Bifidobacterium adolescentis*	100
	B 25	*Bifidobacterium adolescentis*	100
	B 26	*Bifidobacterium adolescentis*	100
	B 27	*Bifidobacterium adolescentis*	100
27	B 30	*Bifidobacterium longum* subsp. infantis	92
30	B 31	*Bifidobacterium adolescentis*	91
32	B 32	No PCR product	
33	B 33	No PCR product	
	B 34	No PCR product	
	B 35	*Bifidobacterium adolescentis*	90
35	B 36	*Bifidobacterium longum*	100
	B 37	*Bifidobacterium longum*	100
	B 38	*Bifidobacterium pseudocatenulatum*	100
		Bifidobacterium catenulatum	100
	B 39	*Bifidobacterium bifidum*	100

Table 10. Genotypic identification of *Bifidobacterium* based on 16S rRNA and ITS regions sequencing. (Continued)

Subjects No.	***Bifidobacterium* isolates**	**Match organism**	**% Identity**
38	B 40	No PCR product	
39	B 41	*Bifidobacterium longum*	99
	B 42	*Bifidobacterium bifidum*	100
	B 43	*Bifidobacterium bifidum*	100
	B 44	*Bifidobacterium longum*	99
40	B 46	*Bifidobacterium longum*	91
41	B 48	*Bifidobacterium adolescentis*	99
42	B 49	*Bifidobacterium longum*	89
	B 50	*Bifidobacterium longum*	100
45	B 51	*Bifidobacterium adolescentis*	98
46	B 52	*Bifidobacterium bifidum*	100
	B 53	No PCR product	
	B 54	*Bifidobacterium bifidum*	100
47	B 56	*Bifidobacterium longum*	91
	B 57	*Bifidobacterium pseudocatenulatum*	100
	B 58	*Bifidobacterium longum*	94
	B 59	*Bifidobacterium bifidum*	100
	B 60	*Bifidobacterium pseudocatenulatum*	100

Table 10. Genotypic identification of *Bifidobacterium* based on 16S rRNA and ITS regions sequencing. (Continued)

Subjects No.	*Bifidobacterium* isolates	Match organism	% Identity
48	B 61	*Bifidobacterium adolescentis*	90
	B 63	*Bifidobacterium adolescentis*	90
50	B 66	*Bifidobacterium longum*	94
51	B 69	*Bifidobacterium longum* subsp. infantis	93
	B 70	*Bifidobacterium longum* subsp. infantis	91
52	B 72	*Bifidobacterium longum*	98
53	B 73	*Bifidobacterium longum* subsp. infantis	92
	B 74	*Bifidobacterium longum*	87
54	B 75	*Bifidobacterium longum* subsp. infantis	91
	B 76	*Bifidobacterium adolescentis*	91
	B 77	*Bifidobacterium adolescentis*	91
55	B 79	*Bifidobacterium adolescentis*	90
61	B 82	*Bifidobacterium longum* subsp. infantis	91
	B 85	*Bifidobacterium longum* subsp. infantis	92
	B 86	*Bifidobacterium adolescentis*	98
62	B 87	*Bifidobacterium adolescentis*	91
	B 89	*Bifidobacterium longum*	89

Table 10. Genotypic identification of *Bifidobacterium* based on 16S rRNA and ITS regions sequencing. (Continued)

Subjects No.	*Bifidobacterium* isolates	Match organism	% Identity
63	B 90	*Bifidobacterium longum* subsp. infantis	92
	B 91	*Bifidobacterium adolescentis*	90
	B 92	*Bifidobacterium adolescentis*	90
	B 93	*Bifidobacterium adolescentis*	91
	B 94	*Bifidobacterium adolescentis*	90
64	B 97	*Bifidobacterium adolescentis*	90
	B 98	*Bifidobacterium bifidum*	100
	B 99	*Bifidobacterium adolescentis*	91
	B 100	*Bifidobacterium adolescentis*	90
	B 101	*Bifidobacterium bifidum*	100
65	B 102	*Bifidobacterium longum*	100
	B 103	*Bifidobacterium longum*	100
	B 104	*Bifidobacterium longum*	100
	B 105	*Bifidobacterium adolescentis*	98
	B 106	*Bifidobacterium longum*	100
66	B 107	*Bifidobacterium longum*	100
	B 108	*Bifidobacterium longum*	99
67	B 109	*Bifidobacterium longum*	90
	B 110	*Bifidobacterium longum*	90

Table 10. Genotypic identification of *Bifidobacterium* based on 16S rRNA and ITS regions sequencing. (Continued)

Subjects No.	*Bifidobacterium* isolates	Match organism	% Identity
68	B 111	*Bifidobacterium longum* subsp. infantis	91
70	B 112	*Bifidobacterium adolescentis*	100
	B 113	*Bifidobacterium longum* subsp. infantis	91
	B 114	*Bifidobacterium adolescentis*	100
	B 115	*Bifidobacterium bifidum*	100
	B 116	*Bifidobacterium adolescentis*	100
71	B 119	*Bifidobacterium longum* subsp. infantis	100
	B 120	*Bifidobacterium longum* subsp. infantis	100
	B 121	*Bifidobacterium longum* subsp. infantis	91
	B 122	*Bifidobacterium longum* subsp. infantis	93
72	B 124	*Bifidobacterium longum* subsp. infantis	100
	B 125	*Bifidobacterium longum* subsp. infantis	100
	B 126	*Bifidobacterium longum* subsp. infantis	100
	B 127	*Bifidobacterium longum* subsp. infantis	100
	B 128	*Bifidobacterium longum* subsp. infantis	100
	B 129	*Bifidobacterium longum* subsp. infantis	100
73	B 130	*Bifidobacterium longum*	99
	B 131	*Bifidobacterium longum*	99
76	B 132	*Bifidobacterium adolescentis*	90
	B 133	*Bifidobacterium adolescentis*	91

Table 11. Genotypic identification of *Lactobacillus* based on 16S rRNA gene dideoxy sequencing.

Subjects No.	***Lactobacillus* isolates**	**Match organism**	**% Identity**
14	L 1	*Lactobacillus gasseri*	100
23	L 2	*Lactobacillus gasseri*	100
27	L 3	*Lactobacillus gasseri*	100
29	L 4	*Streptococcus* sp.	100
	L 5	*Lactobacillus gasseri*	100
30	L 6	*Lactobacillus salivarius*	100
	L 7	*Lactobacillus fermentum*	100
	L 8	*Lactobacillus salivarius*	100
	L 9	*Lactobacillus fermentum*	99
	L 10	*Lactobacillus gasseri*	100
32	L 11	*Lactobacillus salivarius*	100
34	L 12	*Lactobacillus fermentum*	100
35	L 13	*Lactobacillus ruminis*	99
37	L 14	*Lactobacillus mucosae*	100
	L 15	*Lactobacillus mucosae*	100
42	L 17	*Lactobacillus salivarius*	99
	L 18	*Lactobacillus fermentum*	100
43	L 19	*Lactobacillus vaginalis*	99
	L 20	*Lactobacillus gasseri*	100

Table 11. Genotypic identification of *Lactobacillus* based on 16S rRNA gene dideoxy sequencing. (continued)

Subjects No.	*Lactobacillus* isolates	Match organism	% Identity
46	L 21	*Lactobacillus fermentum*	100
	L 22	*Lactobacillus salivarius*	100
47	L 23	*Lactobacillus salivarius*	100
	L 24	*Lactobacillus mucosae*	100
48	L 25	*Lactobacillus gasseri*	100
50	L 26	*Lactobacillus gasseri*	100
57	L 27	*Lactobacillus oris*	99
	L 28	*Lactobacillus ruminis*	99
62	L 29	*Lactobacillus gasseri*	100
64	L 30	*Lactobacillus gasseri*	100
	L 31	*Lactobacillus rhamnosus*	100
66	L 32	*Lactobacillus gasseri*	100
69	L 33	*Lactobacillus rhamnosus*	100
	L 34	*Lactobacillus rhamnosus*	100
	L 35	*Lactobacillus rhamnosus*	100
71	L 36	*Enterococcus faecalis*	99
75	L 38	*Lactobacillus gasseri*	100
76	L 39	*Lactobacillus casei*	100
	L 40	*Lactobacillus salivarius*	100
	L 41	*Lactobacillus gasseri*	100

CHAPTER V

DISCUSSION

The gastrointestinal tract of the fetus is initially sterile, but microbes from the mother and the surrounding environment colonize the gut of the term infant following delivery until a dense and complex microbiota develops. In full-term vaginally delivered infants, colonization starts immediately after delivery and microorganisms such as enterobacteria and streptococci appear in feces. In addition, the composition of the gut microbiota is influenced by the diet of the infant. Breastfeeding positively influences gut colonization with bifidobacteria, whereas formula-fed infants have been reported to have a more diverse microbiota, including bifidobacteria, bacteroides, clostridia and streptococci[21]. *Bifidobacterium* and *Lactobacillus* are among those microorganisms believed to be beneficial and can contribute to digestion, immune stimulation and inhibition of pathogens [2]. Members of the genus *Bifidobacterium* are present in large numbers in newborn infants and are considered to be of importance in early infancy[21].

This study is the first to explore in our geographic region of number of bifidobacteria and lactobacilli in healthy, full-term Thai infants during the first month of life and to investigate the potential role of feeding methods.

For infant formulas, a specific prebiotic mixture of galacto-oligosaccharides (GOS) and fructo-oligosaccharide (FOS) has been described to stimulate the growth of bifidobacteria and lactobacilli similar to milk oligosaccharides in human breast milk. Prebiotics are defined as non-digestible food ingradients that selectively

stimulate the growth and/or activity of one or more bacteria in the colon and thereby beneficially affect the host. Several reports showed that the supplementation of infant formulas with this specific mixture of GOS and FOS increased the numbers and difference in species of *Bifidobacterium* and the total number of *Lactobacillus*, reduced the number of pathogens, and induced a short-chain fatty acid profile similar to that found in breast-fed infants. Our results also demonstrated that the number of bifidobacteria and lactobacilli in breast-fed infants and mixed-fed infants was not significantly different as shown in Table 7 and Figure 3.

Our results showed the number of approximately 10^8 bifidobacteria and 10^7 lactobacilli in the infant faeces. Most studies reported that *Bifidobacterium* is dominant flora at concentrations of approximately 10^8 CFU/g and *Lactobacillus* is subdominant flora at concentrations of approximately 10^4 CFU/g of the human gastrointestinal tract[51] by cultivated method.

According to culture method, most reports indicated the low *Lactobacillus* colonization rates in infants in Western countries, while some claim that lactobacilli are present in substantial quantities (10^{7-9} CFU/g feces) in infant faeces. Variations in methodology may account for the differences, since lactobacilli are notoriously difficult to identify by traditional biochemical methods. Another explanation may be that the *Lactobacillus* microbiota differ in different geographical areas. For example, lactobacilli are more frequently isolated from stools of Estonian as compared to Swedish infants. [50]

The fecal samples were analyzed with real-time quantitative PCR assay. This molecular approach can be applied to high-throughput analyses with frozen samples. Real-time quantitative PCR analyses overcome many of the limitations of traditional

bacteriologic culture techniques, such as the low sensitivity, the low level of reproducibility because of the multitude of species to be identified and quantified, and the time-consuming aspects of the conventional methods. However, molecular techniques based on amplification of 16S rDNA require that the microbial cells in the sample first be lysed for the extraction of DNA. There is a vast difference in the susceptibility of the cells of different microbial species to lytic procedure.[32] When only 1 lytic method is used, it is unlikely that template DNA for the real time PCR analysis is extracted with equal success from all species. Therefore, we chose to add a mechanical lysis step to the chemical lyses of the Qiagen stool mini kit.

Hartemink and Rombouts (1999) reported that media used for the detection of bifidobacteria can be classified into five different groups; known non-selective media (MRS, Rogosa), media with elective carbohydrates, media with antibiotics, media with propionate and media with elective substance and/or low pH. From the large number of media used, it can be concluded that there is no standard medium for the detection of bifidobacteria. In this study, Columbia medium was modified by the supplement with glucose and cysteine hydrochloride and addition of bromocresol purple for differentiation of acid-producing bacteria. Beerens (1991) and Lee (2003) The modified Columbia medium was useful for selection and subculture of bifidobacteria, moreover it was easy to prepare.

Jackson et al. (2002) compared the culture of lactobacilli on two commonly used media, LAMVAB and Rogosa agar and reported that the growth of two species, *L. acidophilus* and *L. gasseri*, was not supported on LAMVAB medium. Rogosa agar was more likely to support the growth of non-*Lactobacillus* species.Therefore, non-

selective MRS agar was used for the isolation of *Lactobacillus* from infant faeces in this study.

Inhibition of pathogen growth is regarded as an important property for probiotics [110, 174]. In this study, *Bifidobacterium* and *Lactobacillus* were isolated from breast-fed infants and mixed-fed infants to investigate for their antagonistic activities against gastrointestinal bacterial pathogens such as EHEC, EPEC, ETEC, EIEC, *S.* Typhimurium, *Sh. flexneri* and *V. cholerae.* As shown in Tables 8 and 9, 10 isolates of *Bifidobacterium* including B5, B15, B17, B31, B61, B63, B76, B77, B132 and B133 demonstrated strong antagonistic activities against gastrointestinal pathogens with clear zone to *V. cholerae* and *Sh. flexneri* and weak antagonistic activities against EHEC, EPEC, ETEC, EIEC and *S.* Typhimurium. These *Bifidobacterium* strains were suitable for further investigations to characterize their antimicrobial substances. The *Bifidobacterium* B5, B76 and B77 isolated from breast-fed infants and B15, B17, B31, B61, B63, B132 and B133 isolated from mixed-fed infants were all genotypically identified as *B. adolescentis* with about 90% similarity.

Seven *Lactobacillus* isolates including L6, L7, L8, L9, L12, L17, L18 demonstrated weak antagonistic activities against *V. cholerae* with small clear zone. *Lactobacillus* L6, L7, L8, L9 were isolated from the same mixed-fed infant, L12 isolated from formula-fed infant, and L17 and L18 isolated from same breast-fed infant. Genotypic identification demonstrated that these 7 isolates matched with *L. salivarius* 100%, *L. fermentum* 100%, *L. salivarius* 100%, *L. fermentum* 99%, *L. fermentum* 100%, *L. salivarius* 99% and *L. fermentum* 100%, respectively. It has been reported that the inhibitory effects of *Lactobacillus* varied even within the same

species [159, 176]. The inhibitory effects of our isolates were possibly due to combination of organic acids and probably antimicrobial compounds.

Several *Lactobacillus* strains displayed antibacterial activities via the production of organic acids such as lactic acid, acetic acid and propionic acid and other metabolites such as hydrogen peroxide and short chain fatty acids [4]. Also, specific antibacterial compounds such as reuterin produced from *L. reuteri* which showed strong inhibition to several pathogens [88] have been identified [84]. In addition, the other important antimicrobial substances known as bacteriocins were produced by several species of *Lactobacillus*. *L. plantarum* has been reported to produce various types of plantaricins such as plantaricins S and T [177]. Plantaricins S showed inhibitory activity against gram-positive bacteria including *C. tyrobutyricum, Enterococcus faecalis* and *Propionibacterium* spp. No action was observed against gram-negative bacteria [177]. *L. plantarum* 423 produced a plantaricin 423, which showed inhibitory activity to several food spoilage bacteria and food-borne pathogens, including *Bacillus cereus, Clostridium sporogenes, Enterococcus faecalis, Listeria spp.* and *Staphylococcus* spp. [178]. *Lactobacillus acidophilus* TK9201 produced acidocin A which was active against different species of *Enterococcus, Pediococcus, Streptococcus,* and *Listeria monocytogenes* [179], while Lactococcin MMFII produced from *Lactococcus lactis* MMFII was active against different species of *Enterococcus* and *Lactococcus* [180].

Many studies reported that *Lactobacillus* strains from human feces displayed antagonistic activity against several pathogens [4, 121]. *Lactobacillus* strains isolated from feces inhibited *E. coli, Staphylococcus aureus, Yersinia enterocolitica, Bacillus cereus* [4], *Clostridium difficile* and *E. coli* O157:H7 [121]. Some *Lactobacillus* species

inhibited *Clostridium difficile* due to hydrogen peroxide and lactic acid. *Lactobacillus* P93 showed antibacterial activity due to organic acids and antimicrobial peptide productions. [30]

The conservative and polymorphous 16S rRNA and internal transcribed spacer region sequence of about 600 bp of *Bifidobacterium* were used as targets for amplification of products used for sequencing. In this study, the sequencing results of some isolates of *Bifidobacterium* had low similarity (<98% identity) with the most match species. This indicated that these isolates might be new strains or new species which need further investigation. Some bacterial isolates which had cell morphology similar to *Bifidobacterium* but no PCR product should be reamplifed by universal primers for identification of these strains. Benno and Mitsuoka (1986) reported that bifidobacteria appeared after birth and within a week after and the dominant bacterial group in healthy infants were *B. infantis, B. longum* and *B. breve*. [51] In this study, we found *B. longum, B. bifidum, B. adolescentis* and *B. pseudocatenulatum*.

The 16S rRNA gene of *Lactobacillus* about 1,500 bp was composed of both variable and conserved regions. The gene was large enough, with sufficient interspecific polymorphisms, to provide distinguishing and statistically valid measurements [215]. Universal primers are usually chosen as complementary to the conserved regions at the beginning of the gene and at either of the 540-bp region or at the end of the whole sequence [215]. For the species identification, the 16S rRNA gene sequence is much easier to determine and thus has become the new gold standard [216, 217]. In practice, a range of about a 0.5 to 1% difference or 99 to 99.5% similarities is often used for species identification [215, 218]. DNA sequencing of rRNA genes appeard to be sufficient for the identification of most lactobacilli [5]. The

Lactobacillus species that were most commonly found in infant feces were *L. acidophilus, L. salivarius, L. fermentum* and *L. gasseri.* Ahrne et al. (2005) reported that certain *Lactobacillus* species, especially *L. rhamnosus*, thrive in the intestinal flora of breast-fed infants. The *Lactobacillus* species found in this study included *L. gasseri, L. salivarius, L. fermentum, L. ruminis, L. mucosae, L. vaginalis, L. oris, L. rhamnosus* and *L. casei*.

CHAPTER VI

CONCLUSION

Quantification of bifidobacteria and lactobacilli in feces of 30 breast-fed infants and 33 mix-fed infants (infants received breast-milk and formula-milk) were determined by quantitative real-time PCR. The numbers of bifidobacteria and lactobacilli in fecal samples in breast-fed infants and mixed-fed infants were not significantly different.

Bacterial isolation from 65 fecal samples were presumptively identified to genus *Bifidobacterium* and *Lactobacillus*. Five hundred and seven isolates were obtained, 391 isolates were suspected to be *Bifidobacterium* and 116 isolates were suspected to be *Lactobacillus*. One hundred and eleven isolates of suspected *Bifidobacterium* and 39 isolates of suspected *Lactobacillus* were selected to test for their antagonistic activities using agar spot method. The gastrointestinal pathogens used as indicator strains included ETEC, EIEC, EPEC, EHEC, *S.* Typhimurium, *Sh. flexneri* and *V. cholerae*.

Out of 111 *Bifidobacterium* tested organisms, 106 isolates had inhibitory activity with clear zone and microcolonies. Ten isoloates of *Bifidobacterium* including B5, B15, B17, B31, B61, B63, B76, B77, B132 and B133 demonstrated strong antagonistic activities by showing clear zone against *V. cholerae* and *Sh. flexneri* and the lawn of growth as microcolonies against EHEC, EPEC, ETEC, EIEC and *S.* Typhimurium.

Out of 39 *Lactobacillus* tested organisms, 14 isolates had inhibitory activities. Seven isolates of *Lactobacillus* including L6, L7, L8, L9, L12, L17 and L18 demonstrated weak antagonistic activities against *Vibrio cholerae* with small clear zones. The other 7 isolates showed partial inhibition to *Vibrio cholerae*, *Sh. flexneri* and *S.* Typhimurium with the lawn of growth as microcolonies.

Genotypic identification of 111 *Bifidobacterium* and 39 *Lactobacillus* isolates demonstrated that the species of *Bifidobacterium* and *Lactobacillus* found in the feces of Thai infants at the age of 1 month included *B. longum, B. bifidum, B. adolescentis, B. pseudocatenulatum, B. catenulatum L. gasseri, L. salivarius, L. fermentum, L. ruminis, L. mucosae, L. vaginalis, L. oris, L. rhamnosus* and *L. casei*.

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APPENDIX A

MATERIALS AND EQUIPMENTS

1. Materials

- Absolute Alcohol (Merck, Germany)
- Boric acid
- *Bam*H I Restriction Enzyme (Promega, USA)
- Deoxynucleotide triphosphate
- Ethylene diamine tetraacetic acid (EDTA)
- Ethidium bromide
- FastStart Taq DNA polymerase (Roche, Germany)
- GeneJET^{TM} PCR Cloning Kit (Fermentas, USA)
- GeneRuler^{TM} 100bp DNA Ladder Plus (Fermentas, USA)
- Glycerol
- Glucose
- Gaspak
- Isopropanol
- Lysozyme
- LightCycler® FastStart DNA Master^{PLUS} SYBR Green I (Roche, Germany)
- NucleoSpin® Plasmid (Macherey-Nagel GmbH & Co. KG, Duren, Germany)
- Proteinase K
- QIAamp® DNA Stool Mini Kit (Qiagen, Hilden, Germany)
- QIAquick PCR Purification Kit (Qiagen, Hilden, Germany)
- Sodium dodesyl sulfate (SDS)

- Tris base (Sigma, USA)

- Tween 20

2. Equipments

- Anaerobic Chamber (Envimed, England)

- Analytical balance

- Autoclave (Hirayama, Japan)

- Autopipetts (Gilson, France)

- Centrifuge

- Deep Freezer

- Electrophoresis chamber

- Frogger (DAN-KAR CCRP, USA)

- Heat block

- Hot air oven

- Incubator

- Light Microscope (Nikon, Japan)

- LightCycler 2.0 Instrument (Roche, Germany)

- Microcentrifuge (Eppendorf, USA)

- pH meter

- Spectrophotometer : SmartSpec 3000 (BioRad, USA)

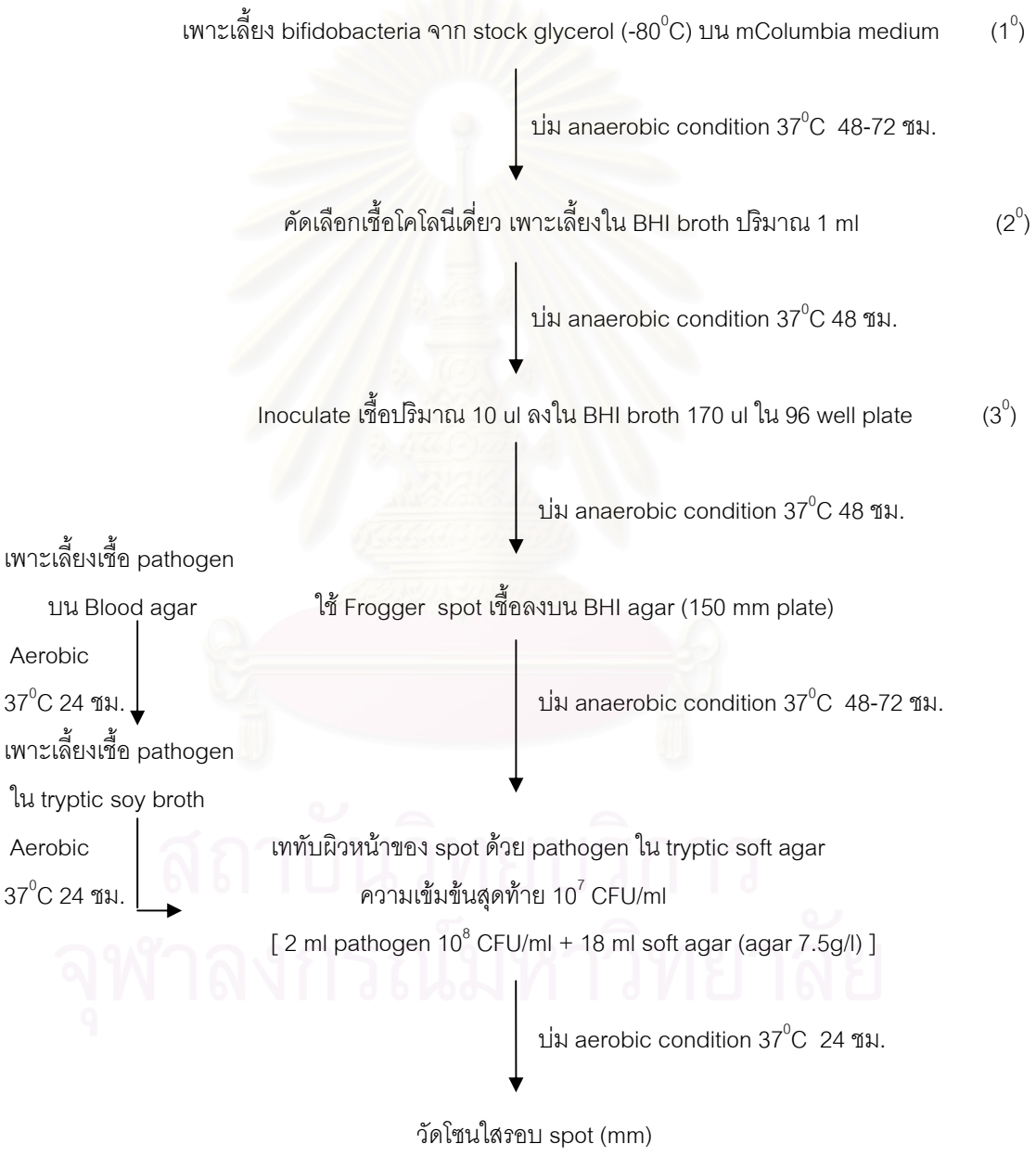
- Thermal cycler

- Vortex mixer

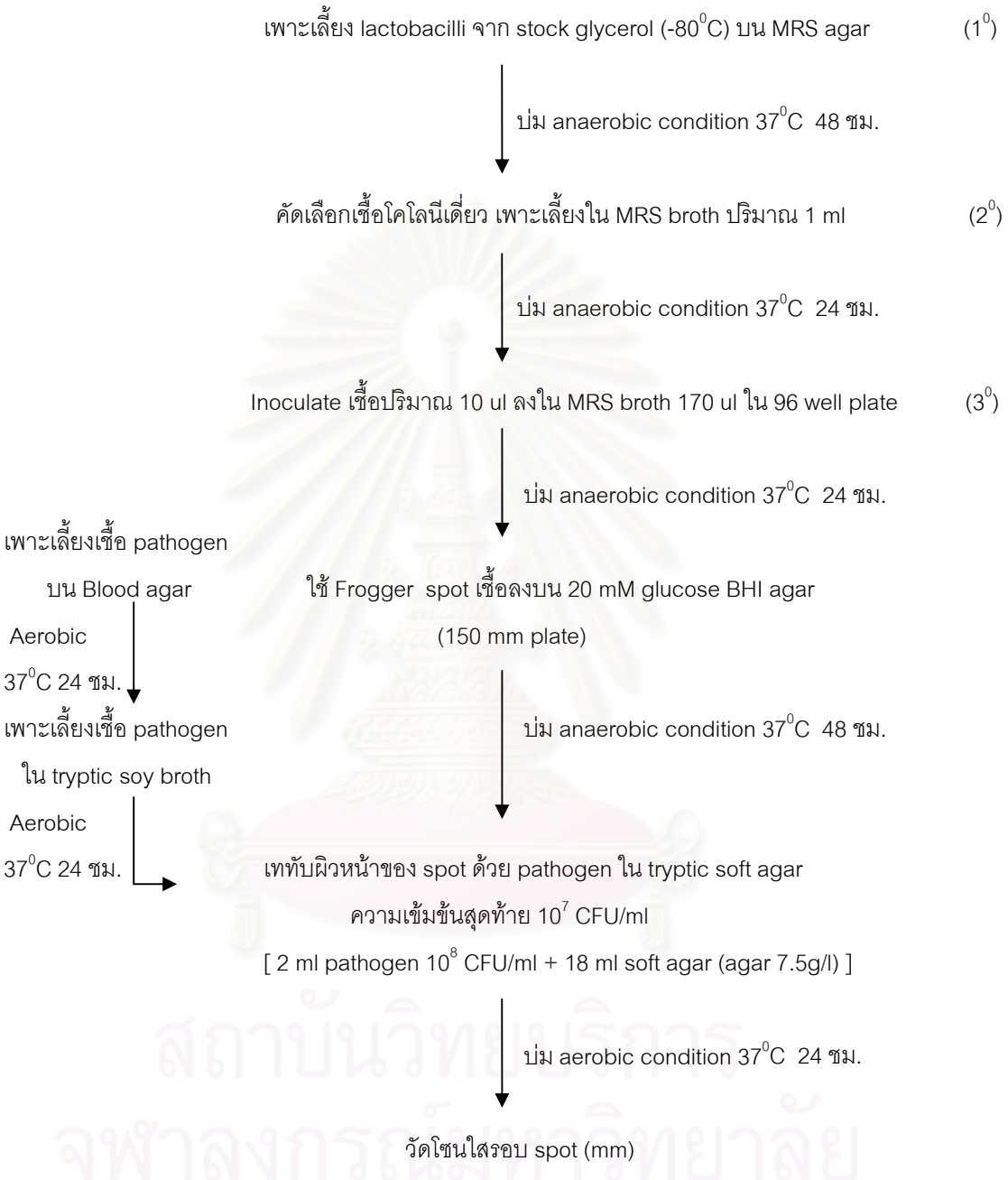
APPENDIX B

FLOW CHART OF PROTOCOL

การทดสอบความสามารถของ Bifidobacteria ในการยับยั้งการเจริญของเชื้อก่อโรค โดย spot method



การทดสอบความสามารถของ Lactobacilli ในการยับยั้งการเจริญของเชื้อก่อโรค โดย spot method



Positive control : *Lactobacillus* sp. No. 9/7

Negative control : MRS broth

APPENDIX C

PREPARATION OF MEDIA

<u>อาหารเลี้ยงเชื้อสำหรับ Bifidobacteria</u>

1. Modified Columbia medium (MC)

Columbia agar base (oxoid)	39	g/L
Glucose	5	g/L
Cysteine hydrochloride	0.5	g/L
Agar	5	g/L
Distil water	1,000	ml

pH 7.3

หากต้องการสังเกตการ ferment น้ำตาลให้เติม <u>bromocresol purple indicator 0.03 g/L</u>

หมายเหตุ : MC ใช้สำหรับการ subculture

2. Brain Heart Infusion Broth (BHB)

Brain Heart Infusion (BBL)	37	g/L
Yeast extract	5	g/L
Cysteine hydrochloride	0.5	g/L
Distil water	1,000	ml

pH 7.2

หมายเหตุ : Brain Heart Infusion Broth ใช้สำหรับการ enrichment

3. Brain Heart Infusion Agar (BHA)

Brain Heart Infusion Agar (BBL)	52	g/L
Yeast extract	5	g/L
Cysteine hydrochloride	0.5	g/L
Distil water	1,000	ml

หมายเหตุ : Brain Heart Infusion Agar ใช้สำหรับการทดสอบ Antagonistic activity

Plate ขนาดใหญ่ใช้ plate ละ 60 ml

4. 20% glycerol BHB

Brain Heart Infusion (BBL)	3.7	g
Yeast extract	0.5	g
Cysteine hydrochloride	0.05	g
Glycerol	20	ml
Distil water	80	ml

หมายเหตุ : 20% glycerol BHB ใช้สำหรับเก็บเชื้อในตู้แช่แข็ง

<u>อาหารเลี้ยงเชื้อสำหรับ Lactobacilli</u>

1. MRS agar

MRS agar (oxoid)	62	g/L
Distil water	1,000	ml

2. MRS broth

MRS broth (oxoid)	52	g/L
Distil water	1,000	ml

3. 20% glycerol MRS broth

ใช้สำหรับเก็บเชื้อในตู้แช่แข็ง เตรียมโดยใช้อัตราส่วน glycerol : MRS broth : DW = 1:2: 2

Glycerol	20	ml
DW	40	ml
MRS broth	40	ml
(MRS 2.08 g + DW 40 ml)		

4. 20 mM Glucose Brain Heart Infusion Agar

Brain Heart Infusion Agar (BBL)	52	g/L
Glucose	1.6	g/L
Distil water	1,000	ml

ใช้สำหรับการทดสอบ Antagonistic activity

APPENDIX D

QUESTIONNAIRE

โครงการวิจัย เรื่อง การหาปริมาณและคุณสมบัติในการยับยั้งเชื้อก่อโรค ของไบฟิโดแบคทีเรีย และแลคโตบาซิลไล ในอุจจาระของเด็กทารกที่กินนมมารดาและทารกที่กินนมผง

ข้อมูลเกี่ยวกับมารดา

1. ชื่อ.....สกุล.....อายุ.....ปี
   ที่อยู่ เลขที่.....หมู่.....ตรอก/ซอย.....ถนน……
   แขวง/ตำบล.....เขต/อำเภอ.....จังหวัด.....
   รหัสไปรษณีย์.....โทรศัพท์บ้าน.....โทรศัพท์มือถือ.....
2. ระยะเวลาในการตั้งครรภ์ (อายุครรภ์) ☐ น้อยกว่า 37 สัปดาห์ ☐ 37-41 สัปดาห์
   ☐ มากกว่า 41 สัปดาห์
3. วิธีการคลอดบุตร ☐ คลอดตามธรรมชาติ ☐ คลอดโดยการผ่าตัด ☐ อื่นๆ .....
4. สถานที่คลอด ☐ ที่บ้าน ☐ ที่โรงพยาบาล ☐ อื่นๆ .....

ข้อมูลเกี่ยวกับทารก

1. ชื่อ.....สกุล.....
2. เกิดวันที่.....เดือน.....พ.ศ. .....อายุ.....เดือน
3. เพศ ☐ ชาย ☐ หญิง
4. ระยะเวลาที่อยู่โรงพยาบาลภายหลังการคลอด ……วัน
5. ชนิดของนมที่ใช้เลี้ยงทารก ☐ นมมารดาเพียงอย่างเดียว
   ☐ นมผงเพียงอย่างเดียว ยี่ห้อ.....
   ☐ นมมารดาและนมผง ยี่ห้อ.....
   ☐ อื่นๆ .....
6. ทารกได้รับประทานอาหารเสริมหรือไม่ ☐ ไม่ได้รับประทาน
   ☐ รับประทานอาหารเสริม คือ .....
7. ทารกเคยได้รับประทานยาปฏิชีวนะหรือไม่ ☐ ไม่เคย ☐ เคย
8. ทารกมีพี่น้องหรือไม่ ☐ ไม่มีพี่น้อง
   ☐ มีพี่.....คน

ผู้กรอกข้อมูล.....วันที่ตอบแบบสอบถาม...../...../.....

APPENDIX E

THE COMPOSITION OF FORMULA MILK

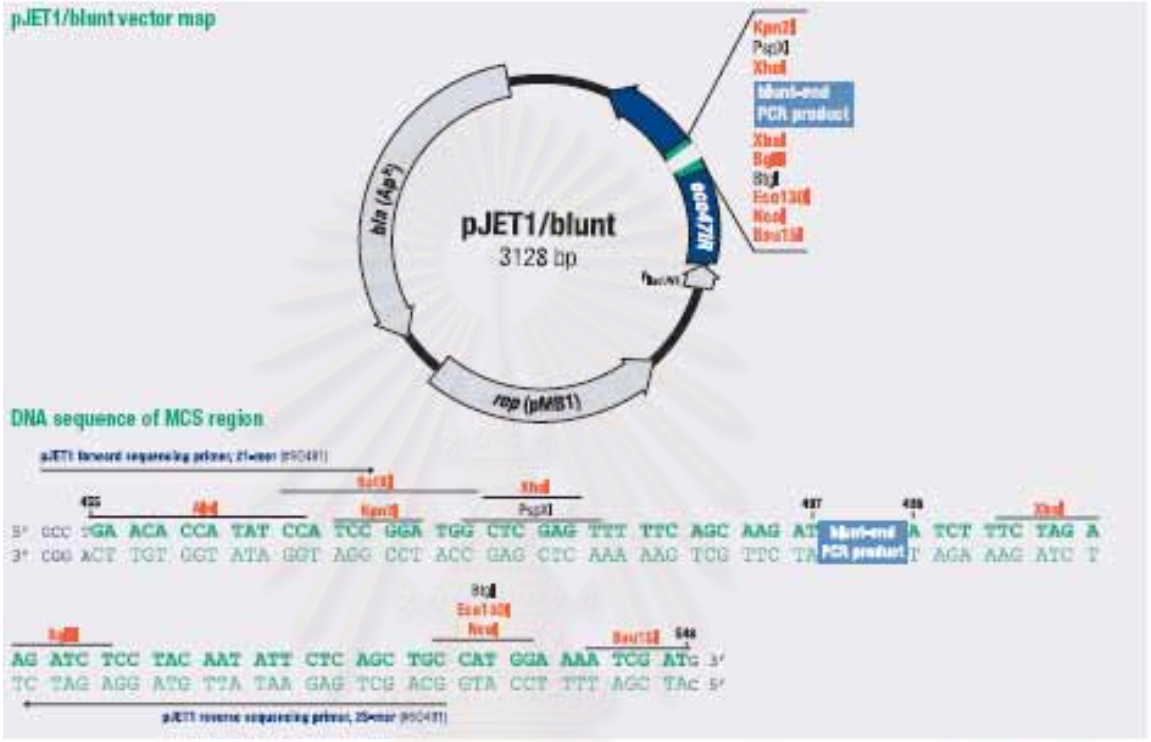
นมผง	ส่วนประกอบ	ราคา
สโนว์ นูโอ	นิวคลีโอไทด์, โอลิโกแซคคาไรด์, ทอรีน, กรดไซอะลิค, โอเมก้า 3 6 9	350 กรัม 125 บาท
ซิมิแลค แอดวานซ์	นิวคลีโอไทด์, ทอรีน, เหล็ก	400 กรัม 162 บาท
เอนฟาแล็ค	DHA, ARA, galactooligosaccharide, innulin	350 กรัม 129 บาท
แล็คโตเย่น	DHA, ARA, omega 3 6 9, vitamin+mineral, iodine, iron, taurine	350 กรัม 107 บาท
แนน 1	นิวคลีโอไทด์, ทอรีน, เหล็ก	400 กรัม 240 บาท
เอส-26	นิวคลีโอไทด์, ทอรีน, เหล็ก, ซิลิเนียม, เบต้าแคโรทีน	350 กรัม 138 บาท
ดูเม็ก	DHA, ARA, oligosaccharide, tryptophan, sialic acid, omega 3 6 9, นิวคลีโอไทด์, ทอรีน	350 กรัม 104 บาท

APPENDIX F

Map and Features of pJET1/blunt Cloning Vector

Available with the GeneJET™ PCR Cloning Kit (#K1221, #K1222)

pJET1/blunt vector map



DNA sequence of MCS region

Genetic elements of the pJET1/blunt cloning vector

Element	Function	Position, bp
rep (pMB1)	A replicon (rep) from the pMB1 plasmid is responsible for the replication of pJET1	1294-1908
Replication start	Initiation of the replication	1306 ±1
bla (Ap^R)	β-lactamase gene conferring resistance to ampicillin. Used for selection and maintenance of recombinant *E.coli* cells	2068-2928
eco47IR	Lethal gene *eco47IR* enables positive selection of the recombinants	187-879
P_{lacUV5}	Modified P_{lac} promoter for expression of the *eco47IR* gene at a level sufficient to provide positive selection without IPTG induction	895-1018
Multiple cloning site (MCS)	Mapping, screening and excision of the cloned insert	545-470
Cloning site	Blunt DNA ends for ligation with insert	497-498
Primer binding sites:		
pJET1 Forward Sequencing	Sequencing of insert, colony PCR	451-471
pJET1 Reverse Sequencing	Sequencing of insert, colony PCR	534-510

Primer sequences

Primer	Sequence
pJET1 Forward Sequencing Primer, 21-mer	5'-GCCTGAACACCATATCCATCC-3'
pJET1 Reverse Sequencing Primer, 25-mer	5'-GCAGCTGAGAATATTGTAGGAGATC-3'

Restriction enzymes that do not cut pJET1/blunt DNA

AarI, Acc65I, AjiI, AjuI, AlfI, BaeI, BclI, BcuI, BoxI, BpiI, Bpu1102I, BseJI, BseRI, BsgI, BshTI, Bsp68I, Bsp1407I, Bst1107I, BstAPI, BtgZI, Cfr42I, CpoI, CspCI, Eco81I, Eco91I, Eco105I, Eco147I, EheI, FalI, FseI, FspAI, KpnI, KspAI, MlsI, MluI, Mph1103I, NdeI, OliI, PacI, PasI, PauI, PdiI, Pfl23II, PsiI, Psp5II, PsrI, PsyI, SanDI, SdaI, SexAI, SfiI, SgfI, SgrAI, SgsI, SrfI, TstI, Van91I, XagI, XcmI, XmaJI.

Enzymes which cut pJET1/blunt DNA once

AdeI	291	Eco72I	173
AleI	455	EcoRI	178
ApaI	1030	FaqI	274
BamHI	1023	GsuI	2226
BbvCI	843	HincII	168
BceAI	1734	HindIII	750
BcgI	2657	Kpn2I	469
BfiI	2186	LguI	1125
BglI	2255	MssI	887
BglII	509	MunI	1018
BpiI	229	Mva1269I	848
Bpu10I	843	NcoI	534
BsaXI	1101	NheI	146
BseYI	1552	NotI	154
Bsp119I	181	NsbI	2361
Bsp120I	1030	PaeI	150
BspTI	141	PdmI	2736
BstXI	466	PfoI	46
Bsu15I	543	Ppu21I	173
BtgI	534	PspXI	477
BveI	390	PstI	1036
CalI	1659	PvuI	2508
Cfr10I	2221	RsaI	2620
Cfr9I	1027	SacI	162
Csp6I	2620	SalI	168
Eam1105I	2136	ScaI	2619
Ecl136II	162	SmaI	1027
Eco130I	534	TatI	2619
Eco31I	2206	XbaI	503
Eco47III	1040	XhoI	478
Eco52I	155	XmiI	168

BIOGRAPHY

Miss Chutima Jittaprasatsin was born on February 10, 1978 in Bangkok, Thailand. She graduated with the Bachelor degree of Science in Microbiology from the Faculty of Science, Srinakharinwirot University in 1999. She is currently working as an medical scientist at the Anaerobic Bacteriology Section, National Institute of Health, Department of Medical Sciences, Ministry of Publish Health, Nonthaburi, Thailand.