



Impacts of Prebiotic-Supplemented Diets and Breastmilk on Population and Diversity of Lactobacilli Established in Thai Healthy Infants

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Abstract

The relative abundance and diversity of lactobacilli present in feces of infants fed with breastmilk and fructooligosaccharide-galactooligosaccharide (FOS-GOS)-, and inulin-galactooligosaccharide (inulin-GOS)-supplemented infant formulae and combination of both were compared. Fecal lactobacilli rapidly colonized and reached maximum total cell counts, which were significantly higher in the infants fed by combining breastmilk with a formula containing either FOS-GOS (C1-A infant) or inulin-GOS (C2-C infant) and the exclusively formula fed ones (F1-F and F2-H infants) than those detected in the exclusively breast-fed (B1-D and B2-E infants) ($P < 0.05$). The greatest relative abundance of fecal lactobacilli species was observed in all infant receiving prebiotic-containing diets, whereas bifidobacteria appeared predominantly in exclusively breast-fed infants. The species composition of lactobacilli was highly unique among individual and more variable in both groups of infants receiving breastmilk than the exclusively formula-fed infants. Breastmilk seem to be a great source of indigenous lactobacilli vertically transferred and continuously seeded infants' gut. Meanwhile, prebiotic supplementation in infant formulae enhanced and sustained the successful colonization of lactobacilli.

Introduction

The early development of gut microbiota during infancy is considered crucial for overall health condition throughout human life [1]. The intestinal microbiota comprise over 35,000 different species with population levels nearly as high as 10^{10} – 10^{12} CFU/g fecal content. The majority of human gut microbiota is obligate anaerobes belonging to Bacteroidetes, Proteobacteria, Actinobacteria, and Firmicutes [2, 3]. Among the Firmicutes phylum, lactobacilli are commonly present and widely distributed in the colon of healthy human host [4]. They play important and beneficial roles in health status of infant apart from bifidobacteria. Lactobacilli have

been well recognized as human commensal microbiota that function to suppress pathogenic bacteria through many mechanisms in preventing and/or treating numerous disorders [5]. Many strains of lactobacilli are commonly used as probiotics, which are defined by the FAO/WHO as live microorganisms that grant health benefit to the host when administered in adequate amounts [6]. Early microbial colonization occurs right after birth with highly unstable and dynamic profiles overtime. The infant gut microbiota becomes more stable and complicated when solid food is introduced. The species composition of infant gut microflora is influenced by multiple interplaying factors among gestational age, delivery mode, hygiene, antibiotic use, country of birth, and feeding regimen/diet [1, 7, 8]. In particular, diet exhibits significant influence in shaping infant gut microbiota by providing substrates essential for bacterial proliferation and function [9]. Several investigators noted the significant differences of intestinal microbiota between breast-fed infants, formula-fed infants, and combination-fed infants contributed to the benefits of breastmilk over formula and breastmilk–formula combination [7, 8, 10, 11].

Lactobacilli are one of the beneficial gut bacteria, which are early developed and established in infant gut. There were significant differences in the abundance of

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fecal lactobacilli among infant groups with different delivery and feeding modes [12, 13]. At the genus level, *Lactobacillus* was detected at higher relative abundance in the feces of breast-fed infants than those of formula-fed and combination-fed ones. Meanwhile, the exclusively formula feeding and combination feeding generally displayed more diverse microbial population and composition [14]. Regarding our previous investigation carried on 31 healthy Thai infants, the average abundance of total fecal lactobacilli was observed at the highest level in vaginally born and exclusively breast-fed infants with conflicting results observed in some individuals. Similarly, the anti-*Salmonella* lactobacilli were detected at the highest number (accounting for 63.8% of total isolates) from the same group of infants at the first week after birth. The most influential factors on gut lactobacilli could be inter-related between delivery modes and feeding regimens [15]. Contrarily, total lactobacillus counts with the highest level in combination-fed and solely formula-fed infants were observed in another set of our investigation with 25 healthy Thai infants. However, more lactobacillus isolates were obtained from combination-fed and exclusively breast-fed infants. Other controversial reports previously indicated that fecal lactobacilli and bifidobacteria showed no association with delivery modes and feeding regimens [16]. Lactobacilli were detected at very low proportion in feces of breast-fed infant [17, 18]. The wide distribution and availability of many prebiotic-supplemented infant

formulae were speculated to nourish the successful establishment of beneficial gut bacteria in infants at early age [18].

Such controversy has roused further search for more perspectives relating to infant supplemented diet. Therefore, the longitudinal study was carried out to preliminarily understand the effect of infant diets (breastmilk, prebiotic supplementation) on the population, diversity, and composition of fecal lactobacilli at the species level in breast-fed, formula-fed, and combination-fed infants. The longitudinal examination can avoid the complication due to the individual uniqueness of strain variation and dynamic shift at each time point. Additionally, analysis of lactobacillus abundance and diversity in maternal breastmilk and infant's feces using FISH and nested PCR-DGGE technique could provide further support to vertical transfer and important source of lactobacilli from mother to infant.

Materials and Methods

Subjects and Sample Collection

This study includes 6 healthy, full-term infants born at 37 to 42 weeks of gestation with no evidence of any sign of disease and no antibiotic treatment at birth (Table 1). Two healthy infants (C1-A and C2-C infants) were fed with a combination diet between breastmilk and an infant formula

Table 1 Characteristics of each infant involved in this study

Infants	Genders	Infant diets	Modes of delivery	Antibiotic treatment	Brands of infant formulae	Age at solid food introduction (months)	Solid food
Infant C1-A	Male	Breastmilk + formula milk	Natural born	No	Brand A (with FOS and GOS)	4	Porridge, pumpkin and banana
Infant C2-C	Male	Breastmilk + formula milk	Cesarean born	Amoxicillin (for 1 week at 3 months of age)	Brand B (with inulin and GOS)	3	Pumpkin, honey and carrot juice
Infant B1-D	Female	Breastmilk	Natural born	No	-	4	Banana
Infant B2-E	Male	Breastmilk	Cesarean born	No	-	4	Porridge and banana
Infant F1-F	Male	Formula milk	Natural born	No	Brand A (with FOS and GOS) 1 week to 2 months of age and Brand C (with <i>B. lactis</i>) after 2 months to 5 months of age	4	Porridge, banana and orange juice
Infant F2-H	Male	Formula milk	Cesarean born	No	Brand B (with inulin and GOS)	4	Banana, pumpkin and boiled egg yolk

either A or B supplemented with fructooligosaccharide-galactooligosaccharide (FOS-GOS) or inulin-galactooligosaccharide (inulin-GOS), respectively. Another two infants (B1-D and B2-E infants) were exclusively fed on breastmilk. The rests (F1-F and F2-H infants) were exclusively fed on the infant formula supplemented with either FOS-GOS (formula A) or inulin-GOS (formula B), respectively. The formula B was switched to the formula C, which was the hypoallergenic one supplemented with *Bifidobacterium animalis* subsp. *lactis* at 2 months old for F1-F infant. The solid foods of fruits, vegetables, and porridge were introduced to all infants of 3–4 months old. Fecal samples were collected once a week until 1 month and every month up to 5 months. To compare bacterial profile between infant feces and breastmilk, healthy mother–infant (full-term) pair was chosen (Table 2). Breastmilk and infant fecal samples were taken from the first week until 10 months after birth. The breastmilk samples were obtained by manual expression after cleaning nipples and areola by wiping with a swab soaked in sterile water and discarding the first drops [17]. The samples collected in sterile plastic containers were then immediately cooled and strictly maintained at 4 °C throughout the transportation period (not longer than 1 h). All fecal samples were immediately processed and kept frozen at – 20 °C.

Enumeration of Dominant Microflora Presents in Infant Feces by Fluorescent In Situ Hybridization (FISH) Technique

The FISH technique for counting specific bacterial group was performed according to Hongpattarakere et al. [19]. The genus-specific 16S rRNA gene oligonucleotide probe (Sigma, St. Louis, USA) targeted for species of *Lactobacillus/Enterococcus* was Lab158 (5'-GGTATTAGCA(T/C)CTGTTTCCA-3'). The others were Bif 164 (5'-CATCCGGCATTACCACCC-3'), Bac 303 (5'-CCAATGTGGGGGACCTT-3'), Lab 158 (5'-GGTATTAGCAYTCCA-3'), Chis 150 (5'-TTATGCGGTATTAATAT(C/T)CCTT-3'), and Eub 338 (5'-GCTGCCTCCCGTAGGAGT-3') targeted to *Bifidobacterium*, *Bacteroides*, *Clostridium histolyticum*, and *Eubacterium* groups, respectively.

Table 2 The conditions of mother–infant pair for this study

Conditions	Mother	Infant
Gestational age	Full-term pregnancy (37 weeks)	Full-term infant (37 weeks)
Delivery mode	–	Natural born
Feeding type	–	The exclusive breastmilk (first day to 10 months)
Antibiotic use	No	No

All probes were labeled with the fluorescent dye Cy3 to facilitate the detection of each group. The nucleotide target 4',6-diamidino-2-phenylindole (DAPI) dye (Sigma, St. Louis, MO, USA) was applied for total bacterial counts. Homogenized infant fecal samples (375 µL) were mixed with 1.125 mL of 4% (w/v) filter-sterilized paraformaldehyde (Acros, New Jersey, USA) and left overnight at 4 °C. The fixed cells were washed twice with cold filter-sterilized phosphate-buffered saline (0.1 M PBS, pH 7.2) (Oxoid, Hampshire, England), and resuspended in 300 µL of PBS: ethanol mixture (1:1 w/v) and stored at – 20 °C until further analysis. The 20 µL of fixed cell suspension appropriately diluted with PBS was dried on TEFLON poly-L-lysine-coated slide (Tekdon, Florida, USA) at 45–50 °C for 15 min. Lysozyme treatment (Sigma, St. Louis, MO, USA) was required for *Lactobacillus/Enterococcus* cells. The slide was dipped into alcohol series (50, 80, and 90% ethanol) (Merck, Darmstadt, Germany) for 3 min at each concentration to facilitate penetration of DNA probe and then finally dried on slide dryer. The 45 µL of hybridization buffer and 5 µL of specific DNA probe solution (50 mg/µL) were mixed and placed on each well. The probe hybridization was allowed at 50 °C for 4 h in hybridization oven (Boekel Scientific, Pennsylvania, USA). The slide was later dipped into washing buffer, (0.9 mmol/l NaCl, 20 mmol/L Tris–HCl, pH 7.2) contained 50 ng/µL DAPI before incubated at the appropriate temperature for 15 min. The slides were then dipped into cold distilled water for 2 to 3 s before air blow-drying. Antifade solution (Fluka, Seelze, Germany) was added onto slide well. The stained cells were enumerated using a fluorescence microscope (Nikon Eclipse 80i, USA) for 15 fields per sample. The numbers of *Lactobacillus/Enterococcus* were determined using the following equation:

$$DF \times ACC \times 7079.68 \times 50 \times DF_{\text{sample}}$$

DF, ACC, DF_{sample} , 7079.68, and 50 represent the dilution factor ($300/375 = 0.8$), the average cell count drawn from 15 microscopic fields of view, dilution of the sample used with a particular probe (e.g., 10× for Lab158 count), the area of the well divided by area of the microscopic field, and conversion factor taking the number based on a milliliter of a sample, respectively.

Analysis of *Lactobacillus* Diversity Using Nested PCR-Denaturing Gradient Gel Electrophoresis (Nested PCR-DGGE) Technique

Total DNA was extracted from either feces or breastmilk according to Walter et al. [20]. Fecal sample (1 g) was washed in 1 mL with PBS to reduce the PCR inhibitors. The pellet of fecal sample was resuspended in 100 µL of sterile Milli-Q water (Omnipur, New Jersey, USA), serially

diluted in 100 μL of 1% Triton X-100 (Fluka, Seelze, Germany), heated at 100 $^{\circ}\text{C}$ for 5 min, and immediately cooled in ice water. The samples were resuspended in 100 μL of lysis buffer (6.7% sucrose (Ajex Finechem, Auckland, New Zealand), 50 mM TrisHCl (pH 8.0) (Amresco, Ohio, USA), 10 mM EDTA (Bio Basic, Ontario, Canada), 20 mg of lysozyme/mL (Sigma, St. Louis, MO, USA), 1000 U of mutanolysin/mL (Sigma, Steinheim, Germany), and 100 mg of RNaseA/mL) (Amresco, Ohio, USA). After incubation for 1 h at 37 $^{\circ}\text{C}$, 6 μL of sodium dodecyl sulfate (20%) (Sigma, St. Louis, MO, USA) and 5 μL of proteinase K (Invitrogen, California, USA), (15 mg/mL) were added and the mixture was further incubated for 15 min at 60 $^{\circ}\text{C}$ to lyse bacterial cells. After cooling on ice, 400 μL of Tris HCl (pH 8.0) was added and the mixture is extracted once with phenol–chloroform–isoamyl alcohol (25:24:1) (Applichem, Darmstadt, Germany). Finally, DNA was precipitated by adding 0.54 volumes of ice-cold isopropanol (Merck, Hohenbrunn, Germany) followed by 30 min at -20°C . The pellet was washed twice with 150 μL 70% ethanol (Merck, Hohenbrunn, Germany), air-dried, and allowed to dissolve overnight in 50 μL TE buffer.

The nested PCR was performed to amplify 16S ribosomal RNA gene of lactobacilli as previously described by Uraipan et al. [21]. In order to increase sensitivity and to facilitate DGGE analysis, nested PCR was performed to amplify target DNA. The first-round PCR was performed with 27-f (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492-r (5'-GGTTACCTTGTTACGACTT-3') as specific primers. The primer of Lac1-f (5'-AGCAGTAGGGAATCTTCCA-3') and Lac2-GC-r (5'CGCCCGGGCGCGCCCGGGCGGC CCGGGGGCACCGGGGGATTYCACCGCTACACATG-3') was applied in the second-round PCR to generate 380 base pair (bp) amplicon of V3 regions. The PCR volumes of 50 μL contained 25 μL red dye PCR master mix (GeNei, Bangalore, India), 5 μL of each primer (2 μM), 1 μL of genomic DNA, and 14 μL of sterile Milli-Q water (Omnipur, Gibbstown, NJ, USA).

DGGE analysis of the PCR amplicons was carried out on the DGGE system (Cleaver Scientific, York, UK). The gels were incubated with 1 \times SYBR[®] Gold (Invitrogen, Grand Island, NY, USA) for 30 min, before being viewed under UV light transillumination (Alpha innotech corporation, San Leandro, USA). The DGGE bands were visualized and cut under UltraBright LED Transilluminator (Gellax, Hsinchu City, Taiwan). Each individual DNA band was purified using PureLink[™] Quick Gel Extraction Kit (Invitrogen, California, USA), before its nucleotide sequence was determined by Ward Medic Ltd., Part. (1st Base Distributor, Thailand). The 16S rRNA gene sequences were compared with those available in (nr/nt) database using BLAST search program at <https://www.ncbi.nlm.nih.gov/>. The percentage of strain variants existing among the same species detected in each

individual was determined using the following equation: $A/B \times 100$ (A was the number of bands appeared to be the same species detected in each infant and B was the total bands detected in each infant.)

Statistical Analysis

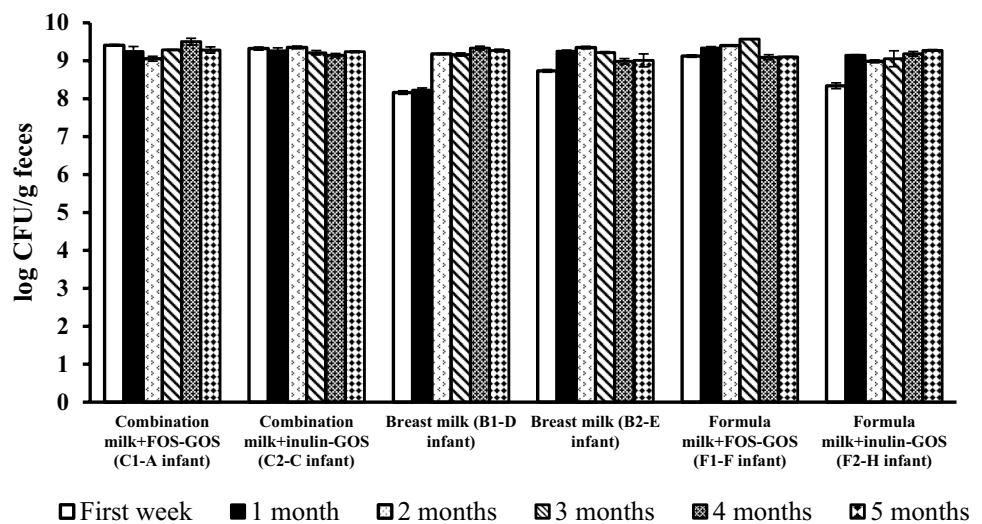
The significant difference of bacterial population was analyzed using SPSS software version 17 for windows. Statistical significance evaluated through Duncan's multiple range test was accepted at $P < 0.05$. Differences in the number of DGGE bands analyzed using parametric ANOVA were declared significant at $P < 0.05$.

Results

The Total Cell Counts and Relative Abundance of Fecal Lactobacilli Present in Infant Fed with Different Diets

The total cell count of fecal lactobacilli was determined and compared between healthy infants fed with breastmilk (B1-D and B2-E infants), infant formulae brand A and B supplemented with FOS-GOS (F1-F infant) and inulin-GOS (F2-H infant), respectively, and combination diet of breastmilk and formulae brand A (C1-A infant) and B (C2-C infant) using FISH techniques. At the first week after birth, the infants fed with combination between breastmilk and a formula supplemented with either FOS-GOS (C1-A infant) or inulin-GOS (C2-C infant) had significantly higher counts of fecal lactobacilli ($P < 0.05$) than the ones fed exclusively on breastmilk and on a formula brand B (Fig. 1). The total cell counts of fecal lactobacilli from infants with the combination diet (C1-A and C2-C infants) and the exclusive formula supplemented with FOS-GOS (F1-F infant) greatly enhanced and rapidly reached a plateau at 10^9 cells/g feces within the first week after birth. In the meantime, total cell count of lactobacilli from the exclusively breast-fed infants (B1-D and B2-E infants) and the exclusively formula supplemented with inulin-GOS (F2-H infant) slowly developed to reach the maximum number of level 10^9 cells/g feces and remained constant throughout 5 months. Lactobacilli appeared to be dominant over bifidobacteria, clostridia, and bacteroides present in feces from combination-fed and exclusively supplementing formula-fed infants with high relative abundance of 20–64% and 21–80%, respectively (Fig. 2). Bifidobacteria were present at the highest relative abundance (32–70%) but lactobacilli were detected at 7–45% in the feces of exclusively breast-fed infants. However, the relative abundance of lactobacilli was rather fluctuated at certain time points at 4 weeks of postpartum in exclusively formula-fed infants (F2-H infant). The prebiotic (FOS-GOS

Fig. 1 The longitudinal total cell counts of lactobacilli present in feces of infants with combination feeding between breastmilk and infant formulae containing either FOS-GOS (C1-A infant) or inulin-GOS (C2-C infant), exclusively breast-feeding (B1-D infant and B2-E infant), and exclusively formula feeding with either FOS-GOS or inulin-GOS supplement (F1-F infant and F2-H infant, respectively) at the first week up to 5 months of infant age. Results are mean values of three replicated \pm SD



and inulin-GOS)-containing formulae seem to significantly enhance early establishment of beneficial lactobacilli and reduced relative abundance of the detrimental genus of *Clostridium* and *Bacteroides*, meanwhile breastmilk seem to promote the abundance of *Bifidobacterium*.

Species/Strain Composition and Diversity of Fecal Lactobacilli from Infants with Different Patterns of Feedings

The nested PCR-DGGE technique targeting V3 region of bacterial 16S rDNA revealed species variation of lactobacilli present in feces of infants fed on three types of diets, including breastmilk, prebiotic-supplementing formulae, and combination of both. The PCR-DGGE profiles of lactobacilli appeared to be highly unique at species level observed in each infant (Fig. 3). The highest number of DGGE bands was observed in infants exclusively fed with the formulae containing FOS-GOS (F1-F infant) and inulin-GOS (F2-H infant). The number of DGGE bands was not different between the infants fed with the combination diets (C1-A and C2-C infants) and the exclusively breast-fed ones (B1-D and B2-E infants) as shown in Table 3. Lactobacilli are established with more variable species in these two groups of infants than the exclusively formula-fed ones (F1-F and F2-H infants). Fecal lactobacilli, including *L. plantarum* (band# 1, 2, 3, 4), *L. salivarius* (band# 10), *L. paracasei* (band# 15), *L. crispatus* (band# 18, 19), and *L. gasseri* (band# 22), were persistently detected throughout 5 month period in the feces from infants fed with the combination diets (C1-A and C2-C infants) and the ones with exclusively breast-fed (B1-D and B2-E infants). Meanwhile, *L. gasseri*, (band# 30, 33) *L. fermentum* (band# 34), and *L. paracasei* (band# 44) remained highly stable in the feces from infants

exclusively fed with both formulae supplemented with inulin-GOS and FOS-GOS.

Thirteen species of *Lactobacillus* were revealed in 6 infants using nested PCR-DGGE technique. About 4–6 species were predominantly detected with high uniqueness to each individual. The most frequently detected *Lactobacillus* species in the feces of Thai healthy infants through the nested PCR-DGGE technique were identified as *L. plantarum*, *L. gasseri*, *L. fermentum*, and *L. paracasei* (Fig. 3). The greatest percentage of strain variants within each species of lactobacilli was detected in the exclusively breast-fed B2-E infant and the F1-F infant with exclusively formula-fed diet supplemented with FOS-GOS (Fig. 4). It is noteworthy to mention that these particular genus appeared as multiple bands in each individual indicating strain differences within the same species as expressed as % strain variants as shown in Fig. 4. FOS-GOS supplementing formula seems to greatly promote and sustain *L. paracasei*, *L. ruminis*, *L. salivarius*, *L. gasseri*, *L. acidophilus*, *L. fermentum*, and *L. rhamnosus*. Meanwhile, the formula supplemented with inulin-GOS enhanced and sustained *L. plantarum*, *L. paracasei*, *Leuconostoc lactis*, *Leuconostoc mesenteroides*, *L. gasseri*, and *L. fermentum*.

At 3 months old, C2-C infant fed with breastmilk and inulin-GOS-supplemented diet was treated with amoxicillin for 1 week. Total count of lactobacilli slightly reduced and certain species (*L. paracasei*) disappeared from DGGE profile (Fig. 3). Only *L. plantarum*, *Leuconostoc lactis* and *Leuconostoc mesenteroides* remained visible with fading bands. Interestingly, all band intensity resumed within 1–2 months after the treatment ended. The dramatic shift of fecal lactobacillus profile was observed at 2 months of age in F1-F infant when the probiotic (*B. lactis*)-supplemented formula C was introduced. Similar to antibiotic treatment, the early colonized strains of lactobacilli could resume and dominate

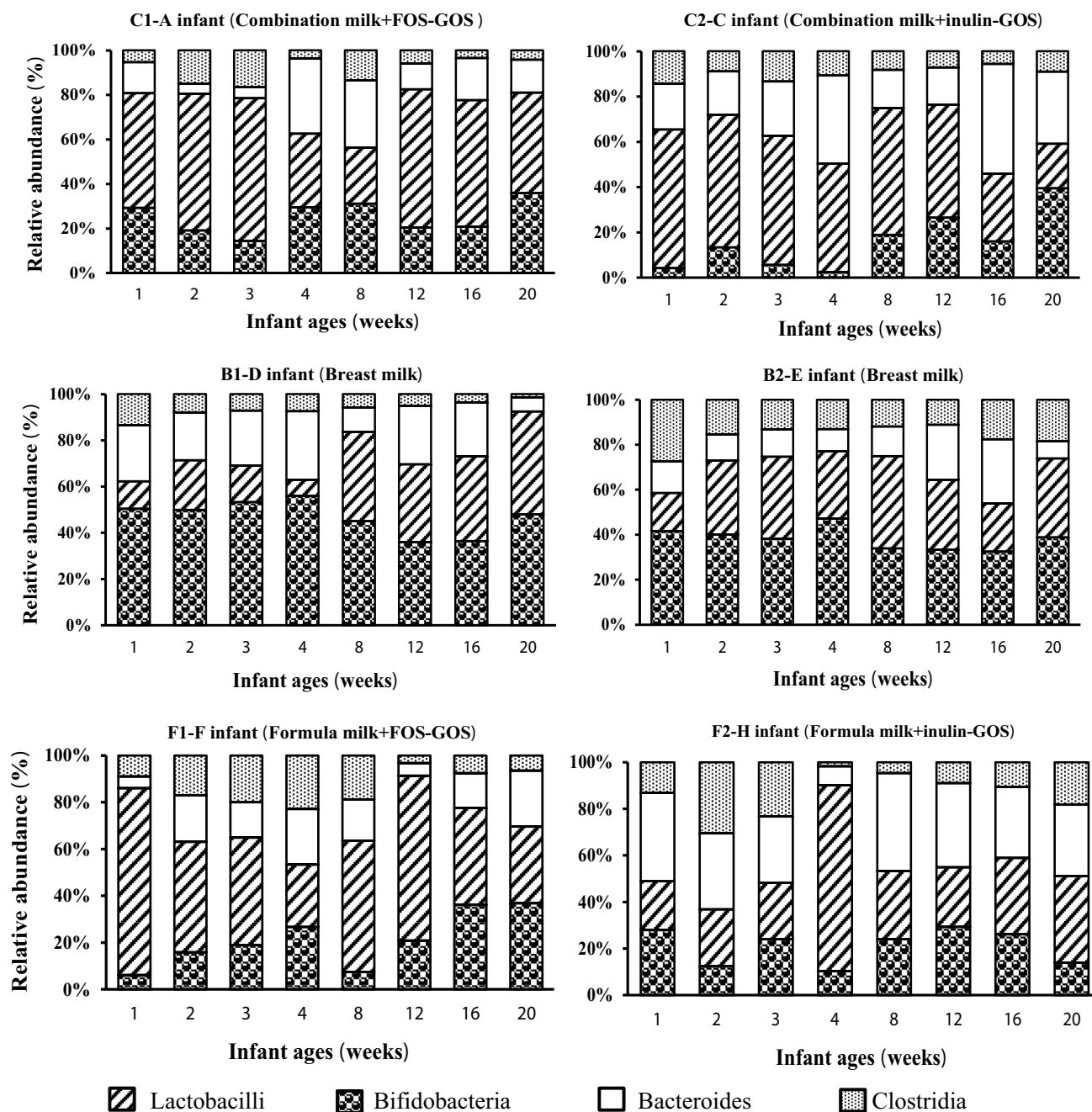


Fig. 2 The relative abundance of fecal lactobacilli, bifidobacteria, bacteroides, and clostridia present in the feces of 6 healthy Thai infants at the first week up to 5 months after birth

afterward although the infant was continuously fed on the formula C. The introduction of solid foods showed strong influence on lactobacillus profile of exclusively breast-fed infants but less influence was observed in the infants with combination and formula feedings.

Comparison of *Lactobacillus* Profiles Between Breastmilk and Infant Feces

The lactobacillus profile of maternal breastmilk clearly corresponded to the one detected in the feces of her infant. The profile of breastmilk was compared with the fecal lactobacillus profile of infant fed on it (Fig. 5). *L. helveticus* (band# 1), *L. plantarum* (band# 4, 5, 6), *L. gasseri* (band# 3, 7, 8, 10, 11), *L. acidophilus* (band# 9), *L. delbrueckii*

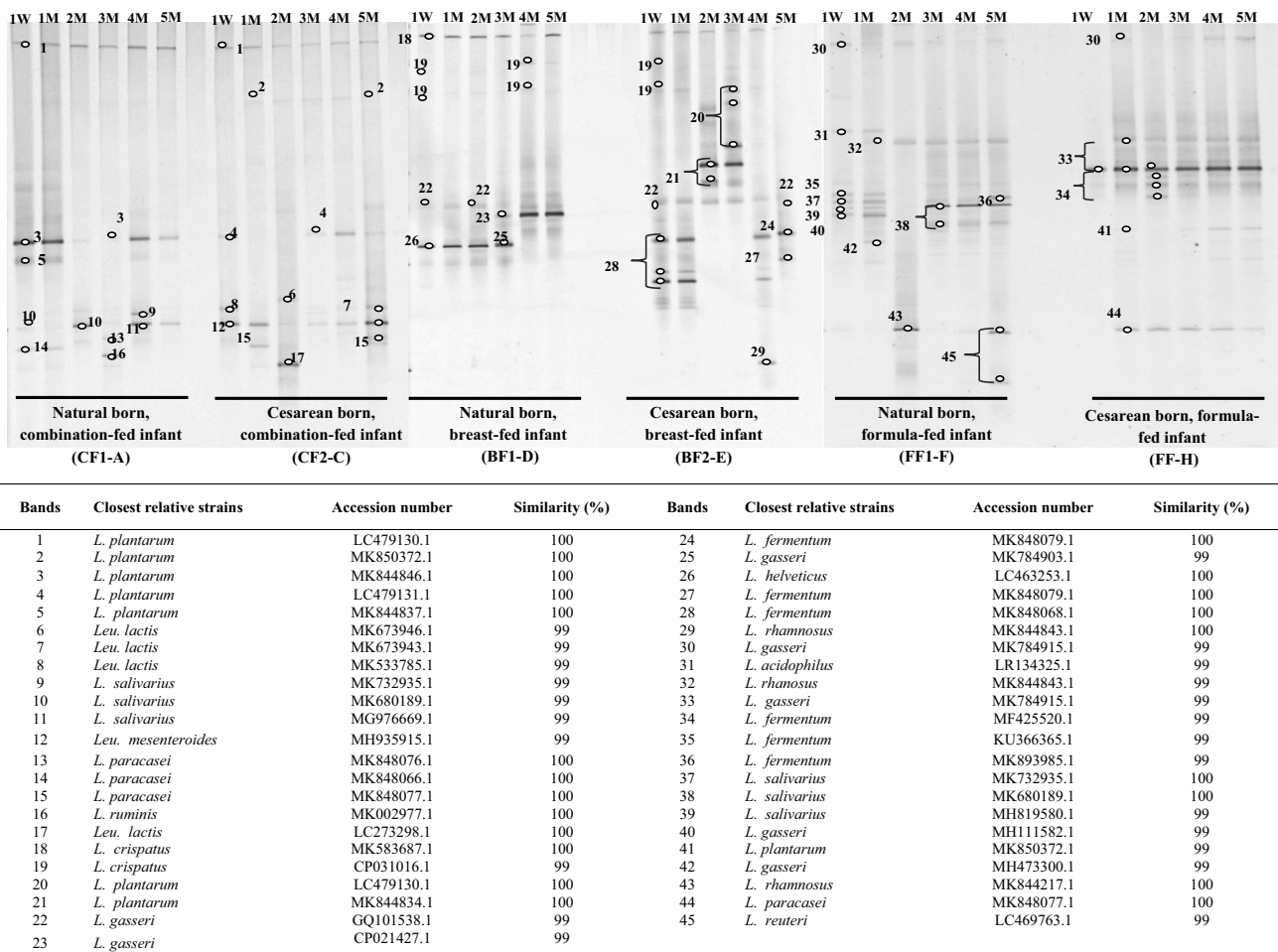


Fig. 3 PCR-DGGE profiles representing lactobacillus species present in feces of infants with combination feeding between breastmilk and infant formulae containing either FOS-GOS (C1-A infant) or inulin-GOS (C2-C infant), exclusively breast-feeding (B1-D infant and B2-E

infant), and exclusively formula feeding with either FOS-GOS or inulin-GOS supplement (F1-F infant and F2-H infant, respectively) at the first week up to 5 months of infant age

Table 3 The number of visible DGGE bands representing lactobacillus species in the feces of 6 healthy Thai infants

Infant ages (months)	Combination milk + FOS-GOS (C1-A)	Combination milk + inulin-GOS (C2-C)	Breast milk (B1-D)	Breast milk (B2-E)	Formula milk + FOS-GOS (F1-F)	Formula milk + inulin-GOS (F2-H)
1 week	8	5	6	8	9	5
1	8	6	6	6	9	8
2	5	8	6	7	3	8
3	5	5	7	7	7	8
4	5	6	5	6	7	8
5	4	8	5	3	7	8
Total	35	38	37	37	42	45

(band# 12, 13), *L. iners* (band# 14), *L. ruminis* (band# 15), *L. murinus* (band# 16), *L. rhamnosus* (band# 17), and *L. casei* (band# 18) were predominantly present in breastmilk. *L. plantarum*, *L. acidophilus*, *L. gasseri*, and *L.*

delbrueckii were the most predominant species frequently detected in breastmilk. These species could also be found in the corresponding infant feces. This result indicated that breastmilk was the important source of indigenous

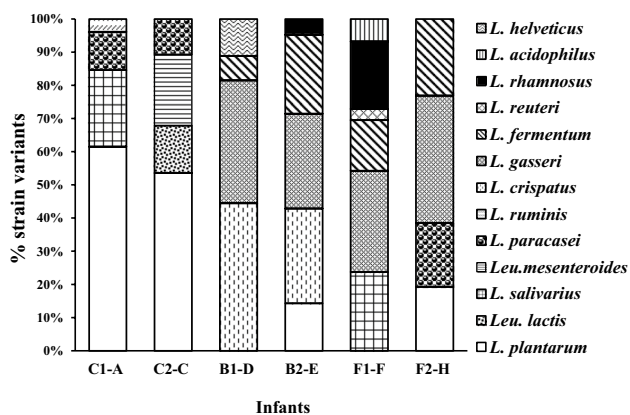
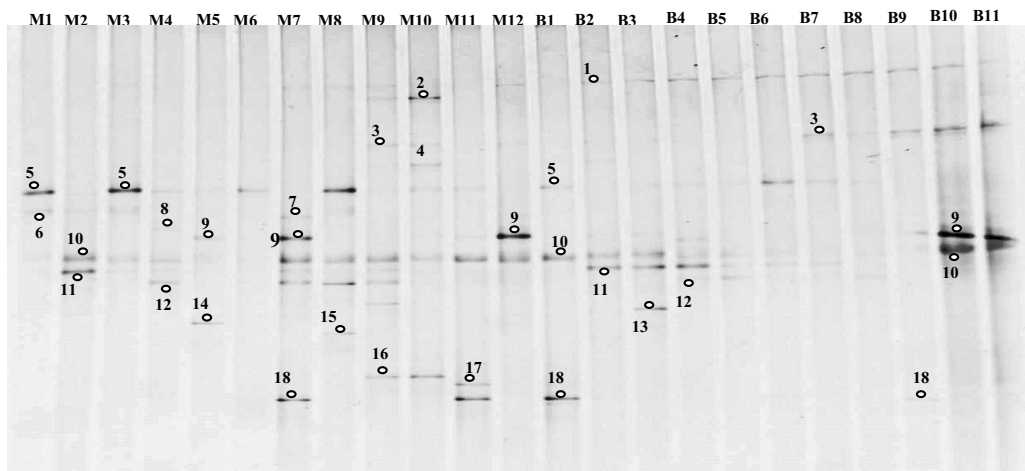


Fig. 4 The percentage of strain numbers among the same species of lactobacilli present in feces of infants with combination feeding between breast milk and infant formulae containing either FOS-GOS (C1-A infant) or inulin-GOS (C2-C infant), exclusively breast-feeding (B1-D infant and B2-E infant), and exclusively formula feeding with either FOS-GOS or inulin-GOS supplement (F1-F infant and F2-H infant, respectively) at the first week up to 5 months of infant age

lactobacilli, which established in infant’s gut and therefore suggesting mother–infant transfer of lactobacilli. This observation supported that breastmilk was the great source of lactobacilli constantly seeded to the infant via breast-feeding.

Discussion

The developing gastrointestinal microbiota in the first year of life is crucial for immune function, nutrient metabolism, and protection from pathogens for the newborn infant. The early establishment and colonization of infant intestinal microbiota is influenced by interrelation of many factors. Diet is one of the most important factors which have a significant influence on the relative proportion of bacteria colonized early in the infant’s gut. Types of diets have been demonstrated to directly modify microbiota composition, by providing the substrates for bacterial proliferation and function, especially in the first day of life when the bacterial population was not yet well established [22]. This study demonstrated that infant diet strongly related to the relative abundance and diversity of fecal lactobacilli. They rapidly increased and reached the highest level within the first week after birth and



Band	Closest relative strains	Accession number	% Similarity	Band	Closest relative strains	Accession number	% Similarity
1	<i>L. helveticus</i>	LC463253.1	100	10	<i>L. gasseri</i>	MK144810.1	99
2	<i>Streptococcus salivarius</i>	MK844212.1	99	11	<i>L. gasseri</i>	MK982458.1	99
3	<i>L. gasseri</i>	MK784915.1	99	12	<i>L. delbrueckii</i>	MK729077.1	100
4	<i>L. plantarum</i>	MK971784.1	100	13	<i>L. delbrueckii</i>	MK784814.1	100
5	<i>L. plantarum</i>	MK850372.1	100	14	<i>L. iners</i>	MK583686.1	99
6	<i>L. plantarum</i>	LC479130.1	99	15	<i>L. ruminis</i>	MK002977.1	99
7	<i>L. gasseri</i>	MK784903.1	99	16	<i>L. murinus</i>	JQ989146.1	99
8	<i>L. gasseri</i>	MK982449.1	100	17	<i>L. rhamnosus</i>	MK844843.1	100
9	<i>L. acidophilus</i>	LR134325.1	99	18	<i>L. casei</i>	JN182264.1	100

Fig. 5 PCR-DGGE profiles of lactobacilli present in breastmilk drawn from a mother at first week up to 11 months (Lane# M1 to M12) of lactation and in feces of her infant at the first week to months after birth (Lane# B1 to B11)

remained stable at the highest abundance throughout the first 5 months, when the infants were fed with combination diets between breastmilk and prebiotic-containing formulae. This is in good agreement with many previous studies showing substantial quantities (10^7 – 10^9 CFU/g feces) of lactobacilli present in infants' feces at early age [23, 24]. The population of lactobacilli colonized in infants' gut increased from 10^6 CFU/g feces at 1st week to 10^8 CFU/g feces at 6 months of age, at which the highest colonization was observed [25]. This study demonstrated that the highest number of lactobacilli was rapidly reached 10^9 CFU/g feces within the first week of life in Thai healthy infant.

The abundance of fecal lactobacilli in relative to bifidobacteria, clostridia and *Bacteroides* appeared at high level and remained quite stable over the whole period of the study in stools of the combination-fed infants (C1-A, C2-C) and the infant exclusively fed with GOS-FOS-supplementing formula (F1-F). These infants had received prebiotic-containing diets. Thus, prebiotic supplementation greatly nourished and rapidly enhanced early establishment of lactobacilli in infants. Likewise, infants exclusively fed with formula supplemented with a mixture of GOS and FOS previously showed higher counts of lactobacilli in feces than the ones fed with non-supplemented formula [18]

Multiple strains of each species, including *L. plantarum*, *L. salivarius*, *L. paracasei*, *L. crispatus*, and *L. gasseri*, were observed in feces of the combination-fed infants and exclusively breast-fed infants. These strains could be indigenous or permanent residents as they remained persistently at the first week up to 5 months. Meanwhile, similar pattern was confined among *L. gasseri*, *L. fermentum*, and *L. paracasei* in exclusively formula-fed infants. The other species of lactobacilli seemed to be transient as they were temporarily detected in this particular group of infant. PCR-DGGE profiles clearly revealed the greatest species variation of lactobacilli in the exclusively breast-fed and combination-fed infants. *Lactobacillus* profiles of these two groups appeared stable from the beginning at the first week of postpartum until the end of study. These species could form rapid establishment to achieve permanent colonization in the infants solely receiving breastmilk although lower relative abundance of lactobacilli was noted. Species/strain composition of fecal lactobacilli was previously noted to be highly unique to each individual by Kimura et al. [26]. The author reported that no common species were detected in different individuals. *L. amylovorus*, *L. mucosae*, *L. plantarum*, *L. gasseri*, *L. fermentum*, *L. delbrueckii*, *L. salivarius*, *L. crispatus*, *L. vaginalis*, *L. brevis*, *L. casei*, *L. acidophilus*, and *L. ruminis* were detected in feces of Japanese adults with multiple strains (differences of PFGE patterns). These strains seemed to be indigenous lactobacilli in Japanese adults [26].

Breastmilk has been recognized as important and continuous source of bifidobacteria and lactobacilli, which are

able to colonize and modulate infant gut [27]. In general, the exclusively breast-fed infant received approximately 800 mL/day of breastmilk containing lactobacilli approximately 10^4 – 10^5 CFU/mL of breastmilk in healthy mothers [28, 29]. The PCR-DGGE profiles of lactobacilli from breastmilk and infant feces strongly confirmed that breastmilk harbored a great deal of lactobacilli which can be transmitted to breast-fed and combination-fed infants. This study demonstrated that lactobacillus species most frequently found in breastmilk were *L. plantarum*, *L. acidophilus*, *L. gasseri*, and *L. delbrueckii*. These species could relatively be detected in the fecal samples of the corresponding infant. The profile of lactobacilli present in breastmilk was quite variable with different band intensity from time to time throughout 12 months of lactation and was corresponded with the fecal profile of her infant.

This result supported that breastmilk was an important source of lactobacilli. This also suggested mother-to-infant transfer of lactobacilli. The vertical lactobacillus transfer constantly continued during the first few months of life as supported by Heikkila and Saris [30]. Lactobacilli residing in the maternal gut could possibly colonize mammary gland through an endogenous route (the so-called entero-mammary pathway), involving maternal dendritic cells and macrophages. Such maternal strains may well adapt to indigenously live and colonize in infant [31]. This study therefore strongly supported the early transfer of indigenous lactobacilli from the mothers to their babies. These strains could be promoted and sustained in the infant's gut depending upon availability of prebiotic substrates either endogenously present in breastmilk or exogenously supplemented in most commercially available infant formulae. Apart from its basic nutritional value, breastmilk contains a high level of complex human milk oligosaccharides (HMOs) at the concentration of 10–15 g/L. HMOs serve as prebiotic component to shape up healthy gut ecosystem by specifically promoting growth of beneficial bacteria, particularly bifidobacteria and lactobacilli in human gut [32]. Multiple species of *Lactobacillus* possess enzymes capable of hydrolyzing HMOs. *L. plantarum*, *L. acidophilus*, *L. reuteri*, and *L. gasseri* widely utilized GlcNAc in HMOs as a sole carbon source [33, 34]. *L. delbrueckii* moderately consumed fucosylated and sialylated HMO [35], and *L. casei* utilized Type II of HMOs [36].

According to this study, the combined feeding between breastmilk and formula milk supplemented with either inulin-GOS or FOS-GOS greatly enhanced lactobacilli in correlating to formula milk supplemented with inulin-GOS or FOS-GOS as confirmed by PCR-DGGE profile. The results confirmed that prebiotic roles of FOS-GOS or inulin-GOS could promote healthy gut microbiota at high proportion and reduce the detrimental ones. This is in agreement with numerous studies demonstrating that mixture of FOS-GOS

or inulin-GOS could stimulate lactobacilli in the similar pattern to milk oligosaccharides present in human milk [22, 37, 38]. Based on the analysis of human milk and high concentration of galactose, a mixture of inulin-GOS and FOS-GOS was developed to mimic human milk for use in infant formulae [22, 39]. Moreover, combination feeding was able to support *L. plantarum* growth and the exclusive feeding with formula supplemented with FOS-GOS and inulin-GOS enhanced *L. gasseri* and *L. fermentum*, which was rather similar to lactobacillus profile of exclusively breast-fed infants. The mixtures of FOS-GOS or inulin-GOS could mimic size distribution of HMO molecules to support optimal growth of both bifidobacteria and lactobacilli [39, 40]. Addition of the specific prebiotic mixture of FOS-GOS or inulin-GOS resulted in a distribution of the different *Lactobacillus* species similar to those found in breast-fed infants [22]. Likewise, several studies demonstrated that lactobacillus species appear to be rather similar. *L. acidophilus*, *L. gasseri*, *L. johnsonii*, *L. reuteri*, *L. paracasei*, *L. rhamnosus*, *L. plantarum*, and *L. fermentum* were the most common species present in breast-fed and formula-fed infants [24, 41]. *L. gasseri* and *L. fermentum* were most commonly found in feces of Japanese volunteers [26].

In fact, the prebiotic mixture, which contains low as well as high molecular mass oligosaccharides, was designed to create optimal growth conditions for both bifidobacteria and lactobacilli [42]. Many strains of lactobacilli such as *L. reuteri*, *L. rhamnosus*, *L. fermentum*, *L. gasseri*, and *L. plantarum* greatly utilized GOS by producing β -galactosidase to hydrolyze GOS [43]. Multiple strains of *Lactobacillus* such as *L. plantarum*, *L. gasseri*, and *L. rhamnosus* produce extracellular enzymes for FOS hydrolysis when exposed to FOS [44]. In addition, a minority of *Lactobacillus* was able to grow on inulin [45]. However, this study found that *L. plantarum*, *L. gasseri*, *L. fermentum*, and *L. paracasei* were persistent and predominant in CF2-B and FF2-H infants which received formula milk supplemented with mixed GOS-inulin. *L. paracasei*, *L. plantarum*, and *L. gasseri* were previously demonstrated to ferment inulin as a sole energy source [46, 47].

As mentioned previously, the species composition of lactobacilli was unique among individual. This must depend on bacterial sources, at which the infants could acquire for lactobacilli. The dominant species found in the exclusively breast-fed infant (B1-D infant) were *L. crispatus* and *L. gasseri*, whereas *L. fermentum*, *L. crispatus*, and *L. gasseri* were dominant species in the other (B1-E infant). This result is related to the composition of breastmilk microbiota, which functioned as a major and continuous source of beneficial bacteria to infants. In general, breastmilk contains a diverse population of bacteria depending on maternal gut microbiota and health status. Consequently, it also provided a postnatal microbial link as its high diversity of microbes could

possibly contribute to infant microbial colonization [48]. Moreover, the shifts of infant gut bacterial community were associated with the proportion of breast-feeding in a dose-dependent manner [49].

The establishment of stable gut microbiota generally accompanied certain transitions that occurred soon after birth, during lactation and during weaning period, at which solid foods were introduced [8]. However, this study demonstrated that many species, including *L. plantarum*, *L. crispatus*, *L. rhamnosus*, *L. fermentum*, *L. paracasei*, and *L. gasseri*, appeared at the first week after birth and constantly persisted thereafter even though the solid foods were introduced. They could also resume after antibiotic treatment and change of infant formula confirming the importance of early establishment at the first week of life. These permanent colonizers were likely true or indigenous species residing in infant gut. *L. plantarum*, *L. acidophilus*, *L. gasseri* and *L. delbrueckii* were the most frequently detected lactobacilli in breastmilk, which could be vertically passed to infants. Moreover, the infants receiving the combination feeding harbored multiple strains of *L. plantarum* compared with other infants. Likewise, multiple strains of *L. gasseri* and *L. fermentum* appeared to be enriched in both infants exclusively fed on FOS-GOS- and inulin-GOS-containing formulae. This provided preliminary information that breastmilk could be a major continuous supply for the indigenous strains of lactobacilli, while prebiotic ingredients sustained and nourished their abundance. This also depended on the ability of such strains to utilize the prebiotics available in either breastmilk or commercial infant formulae.

Conclusion

The rapid development of lactobacilli was noted in the infants with combination diets between breastmilk and infant prebiotic-supplementing formulae. Breast-feeding and combination feeding have promoting effect on growth and persistency of certain lactobacillus species in Thai healthy infants. These included *L. plantarum*, *L. fermentum*, *L. crispatus*, *L. paracasei*, and *L. gasseri*, which were dominant with multiple strains. They were most likely acquired from breastmilk, which constantly fed to infants. This preliminary result suggested the combination feeding could provide infants with potential indigenous lactobacillus species present in breastmilk, apart from natural prebiotic HMOs. Thus, breastmilk could be claimed as natural source of synbiotic component. Meanwhile, the prebiotic-supplementing infant formulae enhance and sustain their growth and persistency in infant gut. This preliminary result can lead to further investigation performing on larger group of Thai infants to confirm and support the efficacy of breastmilk, prebiotic,

probiotic-containing diets in combination of breastmilk on the abundance and diversity of lactobacilli at the species level and to understand their influence on maintaining good gut bacterial profile once antibiotics are introduced.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical Approval This research protocol was reviewed and approved by the Ethics Committee, Faculty of Medicine, Prince of Songkla University (EC Number: 55–244–19–2–3) and (EC Number: 55–243–19–2–3). Verbal informed consent was obtained from all infants' parents on behalf of the participating baby. The verbal consent was granted by the Ethics Committee because this research presented negligible risks to the infant participants. Moreover, all samples and data were processed and analyzed anonymously.

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