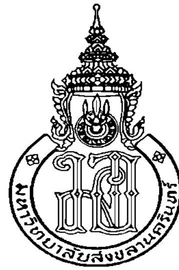

# Biodiversity of Beneficial Bacteria in Infant Feces and Their Potential Probiotic Functions

Khanitta Kongnum

A Thesis Submitted in Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Biotechnology

Prince of Songkla University

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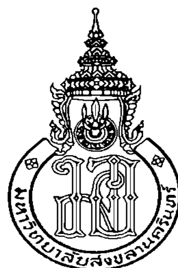


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**Thesis Title** Biodiversity of Beneficial Bacteria in Infant Feces and Their Potential Probiotic Functions

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**Major Program** Biotechnology

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Dean of Graduate School

This is to certify that the work here submitted is the result of the candidate’s own investigations. Due acknowledgement has been made of any assistance received.

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I hereby certify that this work has not been accepted in substance for any degree, and is not being currently submitted in candidature for any degree.

..................................................Signature
(Miss Khanitta Kongnum)
Candidate

**หัวข้อวิทยานิพนธ์** ความหลากหลายของแบคทีเรียที่มีประโยชน์ในอุจจาระเด็กทารกและศักยภาพการเป็นโปรไบโอติก

**ผู้เขียน** นางสาวขนิษฐา คงนุ่ม

**สาขาวิชา** เทคโนโลยีชีวภาพ

**ปีการศึกษา** 2560

## บทคัดย่อ

Bifidobacteria ในทางเดินอาหารเด็กทารกแรกคลอดมีความสำคัญต่อสุขภาพของเด็กทารก ซึ่งมีหลายปัจจัยที่ส่งผลต่อการยึดครองพื้นที่ของ bifidobacteria ในทางเดินอาหารเด็กทารก ได้แก่ วิธีการคลอด อายุของเด็กทารก การได้รับยาปฏิชีวนะ และชนิดของนมที่เด็กทารกได้รับ ศึกษาปัจจัยที่มีผลต่อประชากร bifidobacteria, clostridia, bacteroides, eubacteria และแบคทีเรียทั้งหมด โดยใช้แบบแผนการทดลองทางสถิติ Factorial experimental design ซึ่งปัจจัยของอายุเด็กทารกและชนิดของนมที่เด็กทารกได้รับ เป็นปัจจัยที่มีผลต่อประชากร bifidobacteria, clostridia, bacteroides และ eubacteria ขณะที่ปัจจัยของการคลอดและชนิดของนมเป็นปัจจัยที่มีผลต่อประชากรแบคทีเรียทั้งหมด โดยพบว่าจำนวนของ bifidobacteria ในเด็กทารกทั้งหมด เพิ่มขึ้นอย่างรวดเร็ว ในสัปดาห์แรกจนถึง 2 เดือน ของอายุเด็กทารก และจะคงที่หลังจาก 2 เดือน ของอายุเด็กทารก ในขณะที่จำนวนของ clostridia, bacteroides และ eubacteria จะเพิ่มขึ้นจากสัปดาห์แรกจนถึง 5 เดือน ของอายุเด็กทารก นอกจากนี้การได้รับนมแม่เพียงอย่างเดียวสามารถสนับสนุนการเจริญของ bifidobacteria โดยพบว่าในเด็กทารกที่คลอดแบบธรรมชาติและได้รับนมแม่เพียงอย่างเดียว (BF1-D) และเด็กทารกที่ผ่าตัดคลอดและได้รับนมแม่เพียงอย่างเดียว (BF2-E) มีจำนวนสูงของ bifidobacteria กว่าในทารกกลุ่มอื่น และในเด็กทารกที่คลอดแบบธรรมชาติและได้รับนมแม่เพียงอย่างเดียว (BF1-D) มีจำนวนของ bifidobacteria สูงที่สุด คือ 8.79 ถึง 9.83 log CFU/g feces จากสัปดาห์แรกจนถึง 5 เดือน ของอายุเด็กทารก และจำนวน bifidobacteria เพิ่มขึ้น จาก 8.82 เป็น 9.45 log CFU/g feces ในเด็กทารกที่คลอดแบบธรรมชาติและกินนมผงเพียงอย่างเดียว (FF1-F) หลังจากได้รับนมผงที่เติม *Bifidobacterium lactis* ที่ 2 เดือน จนถึง 5 เดือน ของอายุเด็กทารก และการได้รับยาปฏิชีวนะอะม็อกซีซิลลินส่งผลให้จำนวนของ bifidobacteria ลดลง จาก 9.21 เป็น 8.77 log CFU/g feces ในเด็กทารกที่ผ่าตัดคลอดและได้รับนมผสมระหว่างนมแม่และนมผง (CF2-C) ที่ 3 เดือน ของอายุเด็กทารก นอกจากนี้พบว่าการได้รับนมผงเพียงอย่างเดียวจะสนับสนุนการเจริญของ clostridia และ bacteroides โดยพบว่าในเด็กทารกที่ผ่าตัดคลอดและได้รับนมผงเพียงอย่างเดียวมีจำนวนของ clostridia สูงที่สุด คือ 9.22 log CFU/g feces ขณะที่ในเด็กทารกที่คลอดแบบธรรมชาติ

และได้รับนมผสมระหว่างนมแม่และนมผง (CF1-A) และเด็กทารกที่คลอดแบบธรรมชาติและได้รับนมแม่อย่างเดียว (BF1-D) มีจำนวน clostridia น้อยที่สุด นอกจากนี้พบว่าจำนวนของ bacteroides จะเพิ่มสูงขึ้น จาก 8.59 เป็น 9.19 log CFU/g feces ในเด็กทารกที่ผ่าตัดคลอดและได้รับนมผงเพียงอย่างเดียว (FF2-H) และพบว่าในเด็กทารกที่คลอดแบบธรรมชาติและได้รับนมแม่อย่างเดียว (BF1-D) และเด็กทารกที่คลอดแบบธรรมชาติและกินนมผงเพียงอย่างเดียว (FF1-F) มีจำนวนแบคทีเรียทั้งหมดสูงกว่าในเด็กทารกกลุ่มอื่น จากการศึกษากลุ่มประชากร bifidobacteria ด้วยเทคนิค PCR-DGGE พบว่าชนิดของนมที่เด็กทารกได้รับและวิธีการคลอดเป็นปัจจัยที่มีผลต่อเปลี่ยนแปลงประชากร bifidobacteria โดยพบว่าเด็กทารกที่คลอดแบบธรรมชาติและได้รับนมแม่เพียงอย่างเดียว (BF1-D) และเด็กทารกที่คลอดแบบธรรมชาติและได้รับนมผสมระหว่างนมแม่และนมผง (CF1-A) มีจำนวนแถบดีเอ็นเอของ bifidobacteria มากที่สุด นอกจากนี้ในเด็กทารกที่คลอดแบบธรรมชาติและกินนมผงเพียงอย่างเดียว (FF1-F) และ) และเด็กทารกที่คลอดแบบธรรมชาติและได้รับนมผสมระหว่างนมแม่และนมผง (CF1-A) ตรวจพบจำนวนสายพันธุ์ของ bifidobacteria มากที่สุด *Bifidobacterium adolescentis, Bifidobacterium* sp., *Bifidobacterium longum, Bifidobacterium catenulatum* and *Bifidobacterium breve* สามารถคงอยู่ในทางเดินอาหารของเด็กทารกที่คลอดแบบธรรมชาติและได้รับนมแม่เพียงอย่างเดียว (BF1-D) และเด็กทารกที่คลอดแบบธรรมชาติและได้รับนมผสมระหว่างนมแม่และนมผง (CF1-A) นอกจากนี้พบว่า *Bifidobacterium* sp. และ *B. longum* สามารถตรวจพบได้โดยทั่วไปในทางเดินอาหารของเด็กทารกสุขภาพดี

ศึกษาจำนวนประชากรและความหลากหลายของแบคทีเรียแลคติกในเด็กทารกที่ได้รับนมแม่เพียงอย่างเดียว (BF1-D และBF2-E) ในเด็กทารกที่ได้รับนมผสมระหว่างนมแม่และนมผง (CF1-A และCF2-C) และเด็กทารกที่ได้รับนมผงเพียงอย่างเดียว (FF1-F และFF2-H) พบว่า เด็กทารกที่ได้รับนมผสมระหว่างนมแม่และนมผงที่เติมฟรุคโตโอลิโกแซคคาไรด์ และ กาแลคโตโอลิโกแซคคาไรด์ (CF1-A) และเด็กทารกที่ได้รับนมผสมระหว่างนมแม่และนมผงที่เติมอินนูลิน และ กาแลคโตโอลิโกแซคคาไรด์ (CF2-C) มีจำนวนแบคทีเรียแลคติกสูงกว่าในเด็กทารกที่ได้รับนมแม่เพียงอย่างเดียว (BF1-D และBF2-E) และเด็กทารกที่ได้รับนมผงเพียงอย่างเดียว (FF1-F และFF2-H) จากการทำ PCR-DGGE พบว่าจำนวนแถบดีเอ็นเอของแบคทีเรียแลคติกไม่มีความแตกต่างกันในเด็กทารกที่ได้รับนมผงเพียงอย่างเดียว (FF1-F และFF2-H) เด็กทารกที่ได้รับนมผสมระหว่างนมแม่และนมผง (CF1-A และ CF2-C) และเด็กทารกที่ได้รับนมแม่อย่างเดียว (BF2-E) นอกจากนี้พบว่าประเภทของนมที่เด็กทารกได้รับไม่มีผลต่อความหลากหลายของสายพันธุ์แบคทีเรียแลคติก โดยตรวจพบแบคทีเรียแลคติกจำนวน 14 สายพันธุ์ จากตัวอย่างอุจจาระของเด็กทารกทั้งหมด 6 คน ซึ่งในแต่ละเด็กทารกตรวจพบแบคทีเรียแลคติกจำนวน 4-6 สายพันธุ์ โดยสามารถตรวจพบสายพันธุ์

แบคทีเรียแลคติกมากที่สุดในเด็กทารกที่ได้รับนมแม่เพียงอย่างเดียว (BF2-E) และเด็กทารกที่ได้รับนมผงเพียงอย่างเดียว (FF1-F) นอกจากนี้พบว่า uncultured bacteria สามารถตรวจพบได้โดยทั่วไปในทางเดินอาหารของเด็กทารกและการได้รับนมผสมระหว่างนมแม่และนมผงสามารถสนับสนุนการเจริญของ *L. plantarum* และ *Leu. mesenteroides* การได้รับนมผงที่เติมฟรุคโตโอลิโกแซคคาไรด์สามารถสนับสนุนการเจริญของ *L. rhamnosus และ L. gasseri* ในขณะที่การได้รับนมผงที่เติมอินนูลินและกาแลคโตโอลิโกแซคคาไรด์สนับสนุนการเจริญของ *L. gasseri, L. fermentum และ L. paracasei* นอกจากนี้การได้รับนมแม่สามารถสนับสนุนการเจริญของ *L. gasseri*, *L. crispatus*, *L. plantarum* และ *L. helveticus*.

ศึกษาคุณสมบัติการลดปริมาณคอเรสเตอรอลของแบคทีเรียแลคติกจำนวน 30 สายพันธุ์ และbifidobacteria จำนวน 4 สายพันธุ์ ประกอบด้วย *Enterococcus feacalis* 10 สายพันธุ์ *Enterococcus feacium* 6 สายพันธุ์ *Lactobacillus plantarum* 7 สายพันธุ์ *Lactobacillus casei* 2 สายพันธุ์ *Lactobacillus rhamnosus* 4 สายพันธุ์ *Lactobacillus paracasei* 1 สายพันธุ์ *Bifidobacterium longum* 2 สายพันธุ์ และ *Bifidobacterium bifidum* 2 สายพันธุ์ โดยแบคทีเรียทั้งหมดคัดแยกจากอุจจาระเด็กทารกสุขภาพดีและน้ำนมแม่ จากการทดลองพบว่าแบคทีเรียแลคติกและbifidobacteria ทั้ง 34 สายพันธุ์ สามารถลดปริมาณคอเรสเตอรอลในอาหารเลี้ยงเชื้อ MRS ที่เติม 0.3% เกลือน้ำดีในช่วง 14.39-65.57 µg/ml โดยพบว่า *L. plantarum* 108 สามารถลดปริมาณคอเลสเตอรอลได้สูงที่สุด นอกจากนี้ *Ent. faecalis* จำนวน 9 สายพันธุ์ และ *Ent. faecium* จำนวน 5 สายพันธุ์ สามารถผลิตเอนไซม์ bile salt hydrolase (BSH) โดยการทดสอบบนอาหารแข็ง MRS ที่มีการเติม 0.5% ของ sodium salt of taurodeoxycholic acid (TDCA) นอกจากนี้พบว่า เซลล์พัก (เซลล์แบคทีเรียมีชีวิตละลายในฟอสเฟตบัฟเฟอร์) และเซลล์ตาย (เซลล์ที่ให้ความร้อน) ของ แบคทีเรียแลคติกและ bifidobacteria ทุกสายพันธุ์ สามารถจับกับคอเรสเตอรอล ในช่วง 7.62-19.49 mg/ g dry cell weight และ 3.48-13.65 mg/ g dry cell weight ตามลำดับ จากการศึกษาคุณสมบัติการเกาะติดเซลล์ Caco-2 และ เซลล์ HT-29 พบว่า *Ent. faecium* EMA410.4, *Ent. faecalis* M134 และ *Ent. faecalis* EM17.3 สามารถเกาะติดเซลล์ Caco-2 และ เซลล์ HT-29 ได้ดี จากคุณสมบัติความสามารถในการผลิตเอนไซม์ BSH การลดปริมาณคอเรสเตอรอลในอาหารเลี้ยงเชื้อ MRS และความสามารถจับคอเรสเตอรอลบนผิวเซลล์พักและเซลล์ที่ตาย ของ *Ent. faecium* EMA410.4, *Ent. faecalis* M134 และ *Ent. faecalis* EM17.3 ดังนั้น แบคทีเรียแลคติกทั้ง 3 สายพันธ์ สามารถนำไปประยุกต์ใช้เป็นโปรไบโอติกที่มีศักยภาพลดคอเรสเตอรอล

**Thesis title** Biodiversity of Beneficial Bacteria in Infant Feces and Their Potential Probiotic Functions

**Author** Miss Khanitta Kongnum

**Major Program** Biotechnology

**Academic Year** 2017

## ABSTRACT

Gut colonization of bifidobacteria in early infancy is essential for the well-being of the infant. Several factors have involved in bifidobacterial colonization, such as delivery mode, infant age, antibiotic treatment and feeding type. Factorial experimental design was applied to evaluate the factors including delivery modes (natural born and Cesarean born), feeding types (breast milk, formula milk and combination milk) and ages of infant (1 week, 1 month, 2 months, 3 months, 4 months and 5 months) on the bifidobacterial population and composition of infant feces. Among 6 healthy newborn infants, infant age and feeding type were the most influential factor for the population of bifidobacteria, clostridia, bacteroides and eubacteria, whereas feeding type and delivery mode were the main factors influencing total bacteria population. The amount of bifidobacteria rapidly increased from first week to 2 months of infant age and stable then after 2 months in all of infants. Moreover, the amount of clostridia, bacteriodes and eubacteria significantly increased from the first week up to 5 months of ages in all of infants. Feeding type was also main factor influencing on population of bifidobacteria, clostridia, bacteroides, eubacteria and total bacteria. The exclusive breast-feeding greatly supported the bifidobacterial growth. The level of bifidobacteria in both naturally born, exclusive breast fed infant (BF1-D) and Cesarean born, exclusive breast fed infant (BF2-E) was significantly higher than other infants. However, the highest level of bifidobacteria was found in naturally born, exclusive breast fed infant (BF1-D) from first week to 5 months of ages (8.79 to 9.83 log CFU/g feces). In addition, the amount of bifidobacteria in naturally born, formula fed infant (FF1-F) greatly increase (8.82 to 9.45 log CFU/g feces) when received the exclusive formula milk supplemented with *B. lactis* at 2 months to 5 months of age. Using antibiotic (amoxicillin) in Cesarean born, combination fed infant (CF2-C) was significantly decreased the amount of bifidobacteria (9.21 to 8.77 log CFU/g feces) at 3 months of age. Moreover, formula

milk seemed to enhance the growth of clostridia and bacteroides. The highest level of the clostridia was found in Cesarean born, exclusively formula fed infant (FF1-F) (9.22 log CFU/g feces) when received exclusive formula milk supplemented with FOS and GOS at 2 months of age. However, the amount of clostridia was significantly decreased when received the exclusive formula milk supplemented with *B. lactis*. Conversely, low level of clostridia was found in both naturally born, combination fed infant (CF1-A) and naturally born, exclusively breast fed infants (BF1-D). In addition, the amount of bacteroides was significantly increased (8.59 to 9.19 log CFU/g feces) in Cesarean born, formula fed infant (FF2-H) at the first week to 5 months of age. The amount of total bacteria in naturally born, exclusively breast fed infant (BF1-D) and naturally born, exclusively formula fed infant (FF1-F) was significantly higher than another infants.

PCR-DGGE analysis demonstrated that there was change in the bifidobacterial community composition associated with feeding type and delivery mode. The high numbers of bifidobacteria band were observed in both naturally born, exclusively breast fed infant (BF1-D) and naturally born, combination of breast and formula fed infants (CF1-A). However, the highest number of bifidobacterial species was observed in both naturally born, exclusively formula fed infant (FF1-F) and naturally born, combination of breast and formula fed infants (CF1-A). Several species of bifidobacteria including *Bifidobacterium adolescentis, Bifidobacterium* sp. *Bifidobacterium longum, Bifidobacterium catenulatum* and *Bifidobacterium breve* were found persistently from the first week to 5 months of ages in naturally born, both of breast fed (BF1-D) and combination fed infants (CF1-A). The most common bifidobacterial species found in healthy infants were *Bifidobacterium* sp. and *B. longum.*

Feeding type in infancy is the most significant determinant in shaping the profile of intestinal microbiota at early life. The population level and diversity of lactic acid bacteria (LAB) present in the feces of infants were compared between breast-, formula- and combined-feeding types. There were two infant formulae with FOS-GOS and inulin-GOS supplementations. The number of fecal LAB from the infants fed with the combination diet of breast milk and a formula containing FOS-GOS (CF1-A infant) and inulin-GOS (CF2-C infant) was significantly higher than

that from both exclusively breast-fed (BF1-D infant and BF2-E infant) and exclusively formula fed ones (FF1-F infant and FF2-H infant) (*P*<0.05). According to PCR-DGGE analysis, the band richness of LAB population in infants with combination (CF1-A infant and CF2-C infant) and exclusive formula feeding (FF1-F infant and FF2-H infant) and the exclusively breast-fed infants (BF2-E) was not different, while the number of LAB in exclusively breast fed infant (BF1-D) was lower than another. Type of feeding had not a significant influence on LAB diversity. Fourteen species of LAB were found from 6 infants and each infant presented 4-6 species of LAB and the most number of LAB species was found in the infant with the exclusive breast fed infant (BF2-E infant) and exclusive formula fed diet supplemented with FOS-GOS (FF1-F infant). Moreover, uncultured bacteria were commonly found in the commensal intestinal microbiota in Thai infants, independently on feeding type. They were detected abundantly in feces of all infants. Combination feeding was able to support the growth of *L. plantarum* and *Leu. mesenteroides.* The exclusive feeding with formula supplemented with FOS-GOS enhance growth of *L. rhamnosus* and *L. gasseri*, while the formula supplemented with inulin-GOS sustained growth of *L. gasseri*, *L. fermentum* and *L. paracasei.* In addition, exclusive breast milk feeding was capable to promote the growth of *L. gasseri*, *L. crispatus*, *L. plantarum* and *L. helveticus*.

Thirty strains of lactic acid bacteria (LAB) and four strains of bifidobacteria isolated from healthy infant feces and breast milk were evaluated for cholesterol-lowering activity through various mechanisms. *Enterococcus feacalis* (10), *Enterococcus feacium* (6), *Lactobacillus plantarum* (7), *Lactobacillus casei* (2), *Lactobacillus rhamnosu*s (4), *Lactobacillus paracasei* (1), *Bifidobacterium longum* (2) and *Bifidobacterium bifidum* (2) were determined for the ability to assimilate cholesterol from growth media, bile salt hydrolase activity and cholesterol binding property. All of 34 strains were able to assimilate cholesterol in the 0.3 % oxgall bile containing medium in the range of 14.39-65.57 µg/ml using *O*-phthalaldehyde method. *L. plantarum* 108 displayed the highest assimilation. The bile salt hydrolase (BSH) activity was only confined among nine strains of *Ent. faecalis* and five strains of *Ent. faecium* by showing precipitation zone on MRS agar supplemented with 0.5% (w/v) sodium salt of taurodeoxycholic acid (TDCA). The cholesterol binding ability

of the resting cells (live cells) and dead cells (heat-killed cells) was exhibited within all LAB and bifidobacteria range of 7.62-19.49 mg/g dry weight and 3.48-13.65 mg/g dry cell weight. Furthermore, *Ent. faecium* EMA410.4, *Ent. faecalis* M134 and *Ent. faecalis* EM17.3 strongly adhered to Caco-2 and HT-29 cell lines. According to this study, *Ent. faecium* EMA410.4, *Ent. faecalis* M134 and *Ent. faecalis* EM17.3 had greatly ability to produce BSH enzyme, remove cholesterol from the media during growth, remove cholesterol via binding to cellular surface of resting and dead cell and strongly adhered to Caco-2 and HT-29 cell lines. Therefore, these strains can be potentially applied as a good probiotic candidate for lowering cholesterol level.

# ACKNOWLEDGEMENTS

I would like to extend thanks to the many people, who so generously contributed to the work presented in this thesis. First and foremost, I would like to express my gratitude to my supervisor, Assist. Prof. Dr. Tipparat Hongpattarakere. This work would not have been possible without her encouragement and belief in me. She kindly read and offered detailed advices on the direction and theme of this thesis and my paper. This thesis writing would not have been finished without her responses and corrections.

I would especially like to thank my thesis committee: Assoc. Prof. Dr. Suppasil Maneerat, Assoc. Prof. Dr.Wilaiwan Chotigeat and Assoc. Prof. Dr. Sunee Nitisinprasert for reading and kind providing valuable suggestions.

I would like to thank the Office of the Higher Education Commission, Thailand for financial support this Ph.D. research through the program Strategic Scholarships for Frontier Research Network for the Ph.D. Program Thai Doctoral degree for this research. This research work was also financially supported by Graduate School of Prince of Songkla University, Hat Yai.

I am deeply grateful to my friends in my laboratory and in the Department of Industrial Biotechnology for their enthusiasm, assistance and encouragement. Finally, I would like to express my deepest gratitude to my family and Yap Kit Seong for their love, encouragement and support throughout my study. I would not have been able to complete this work. Their constant love, inspiration, support, and generosity for everything which I received along the way of my time are always deeply grateful.

Khanitta Kongnum

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## ABBREVIATIONS AND SYMBOLS

| | |
|---|---|
| BF | Exclusively breast-fed |
| BSH | Bile salt hydrolase |
| CF | Combination-fed infants |
| DGGE | Denaturing gradient gel electrophoresis |
| DMEM | Dulbecco's modified eagle medium |
| FCS | Fetal bovine serum |
| FF | Formula-fed |
| FISH | Fluorescent in situ hybridization |
| FOS | Fructooligosaccharides |
| GI | Gastrointestinal tract |
| GOS | Galactooligosaccharides |
| HMO | Human milk oligosaccharides |
| LAB | Lactic acid bacteria |
| NEAA | Non-essential amino acids |
| PCR | Polymerase chain reaction |
| TDCA | Sodium salt of taurodeoxycholic acid |

# CHAPTER 1

# INTRODUCTION AND REVIEW OF LITERATURE

## Introduction

The normal flora of the human gastrointestinal tract consists of hundreds of different types of microorganisms which are considered as a biologically important composition of human body. Bacteria residing in gastrointestinal tract (GI) can be grouped according to their degree of pathogenicity. Three groups of bacteria have been generally recognized as beneficial or probiotic, potentially pathogenic and pathogenic ones (Westerbeek *et al*., 2006). The most common anaerobic genera are *Bacteroides*, *Bifidobacterium*, *Eubacterium*, *Fusobacterium*, *Clostridium* and *Lactobacillus*, whereas aerobes are Gram-negative enteric bacteria (such as *Escherichia coli* and *Salmonella* spp.) and Gram-positive cocci (such as *Enterococcus*, *Staphylococcus* and *Streptococcus*) (Noverr and Huffnagle, 2004). The healthy gut microbiota is considered essential for host in terms of intestinal development, homeostasis and protection against pathogenic challenges. Certain investigation even referred gut microbiota as an extra organ of the host (O'Hara and Shanahan, 2006). The important role of gut microbes relates to their metabolic reactions, such as fermentation of non-digestible dietary fiber (resistant starch, some oligosaccharides), biotransformation of conjugated bile acids, degradation of oxalate-based complexes and synthesis of some essential vitamins (such as B12 and K) (Montalto *et al*., 2009).

The neonatal period is crucial for early intestinal colonization, which consequently influences on the adult intestinal microbiota and lifelong (Morelli, 2008). The infant gut is thought to be sterile at birth (Zetterstrom *et al*., 1994; Mackie *et al*., 1999; Tannock, 2000). The critical stage of gut colonization occurs within the first few weeks after birth and is influenced by a variety of factors; including gestational age, delivery mode, hygiene, antibiotic treatment, geographical zone and feeding type (Thompson-Chagoyan *et al*., 2007; Marques *et al*., 2010). In early life, one of the primary drivers shaping the changes that occur during infancy and the adult microbiome structure

is type of feeding (Voreades *et al*., 2014). In addition, the feeding types exhibited significant influence on the relative proportions of bacteria that establish in the infant gut by providing substrates for bacterial proliferation and function (Edwards and Parrett, 2002; Guaraldi and Salvatori, 2012).

Breast milk is a rich and natural synbiotic source as it contains many different strains of beneficial bacteria as well as oligosaccharides that act as a prebiotic. Human milk oligosaccharides (HMO) are capable of improving healthy intestinal microbiota, which consequently increases beneficial microbial metabolites and protects the neonate against pathogenic bacteria. Breast milk promotes the healthy composition of the infant gut microbiota by selectively promoting beneficial gut bacteria that can use HMO as growth substrate, particularly, bifidobacteria and lactic acid bacteria (LAB) (Satokari *et al*., 2002; Chen *et al*., 2007). Moreover, these beneficial bacteria play very important role in many physiological functions in the human intestine (Gibson and Roberfroid, 1995). They contribute to digestion, stimulate the immune system, and inhibit the growth of pathogens (Holzapfel *et al*., 1998). *Lactobacillus fermentum, Lactobacillus casei, Lactobacillus paracasei, Lactobacillus delbrueckii, Lactobacillus gasseri and Lactobacillus plantarum* frequently isolated from infants (Ahrne *et al*., 2005; Haarman and Knol, 2006). Bifidobacteria appear after birth and within a week and eventually become dominant group in healthy breast-fed and formula-fed infants. *Bifidobacterium breve*, *Bifidobacterium longum*, *Bifidobacterium adolescentis*, *Bifidobacterium pseudocatenulatum*, *Bifidobacterium bifidum*, and *Bifidobacterium dentium* were the most prevalent species found in infants (Aires *et al*., 2011).

Breast-fed infants show significantly higher counts of *Bifidobacteria* and *Lactobacillus* and lower counts of *Bacteroides*, *Clostridium*, *Staphylococcus* and *Enterobacteriaceae* as compared with formula-fed infants (Rinne *et al*., 2005; Fallani *et al*., 2010). Therefore, considerable efforts have been attempted to improve the composition of the commercial infant formula by supplementing bovine lactoferrin, probiotic and prebiotic ingredients in order to shape newborns' microflora composition in the similar trend as breast milk does (Fernandez *et al*., 2013). The common prebiotic

oligosaccharides including fructooligosaccharides (FOS), galactooligosaccharides (GOS) and inulin are commonly supplemented in many infant formulae widely available in the market (Moro *et al*., 2002; Vandenplas *et al*., 2002; Fanaro *et al*., 2005; Knol *et al*., 2005; Xia *et al*., 2012; Closa-Monasterolo *et al*., 2013).

Probiotic is defined as living microorganisms, which can elicit positive influence on host health when a sufficient number was administered with high survival in the intestinal ecosystem. A large number of one or more strains of a single species or a mixture of several species, which are common components of the stools from healthy human can function as probiotic (Gismondo *et al*., 1999). The probiotic proliferation helps protecting gastrointestinal tract from invasion of pathogenic or opportunistic bacteria. Furthermore, certain probiotic strains are able to aid food digestion, enhance nutrient bioavailability, produce certain B vitamins, modulate host immune system, produce various digestive and protective enzymes, antimicrobial substances (Holzapfel *et al.*, 1998) and assimilate cholesterol (Liong and Shah, 2006). Nevertheless, the well-characterized strains proven to have clinical health benefit are not numerous. Bacteria belonging to *Bifidobacterium* and *Lactobacillus* are most often proven to be suitable probiotic supplements with many different beneficial properties relating to human health. One approach to positively enhance the composition of intestinal probiotic and their metabolic activity is the introduction of so-called prebiotics, which are defined as non-digestible food ingredients that selectively stimulate the growth and/or activity of one or a limited number of beneficial bacteria in the colon and thus improve host health (Gibson and Roberfroid, 1995).

LAB and bifidobacteria have attracted attention as potential cholesterol-lowering probiotics. Gilliland *et al.* (1990) reported that *Lactobacillus acidophilus* reduced blood cholesterol through reduction of cholesterol absorption and bile acid reabsorption in the intestine by directly breaking down cholesterol and deconjugating bile acid, respectively. Lim *et al.* (2004) suggested that *Streptococcus*, *Lactobacillus* and *Bifidobacterium* isolated from human intestine had an excellent hypocholesterolemic effect. *L. acidophilus* ATCC 43121 could incorporate and removed cholesterol from media into the cellular

membrane during growth (Noh *et al*., 1997). While the hypocholesterolemic effect of certain probiotic strains has been well-documented. Many prebiotic ingredients have also gained increasing attention in cholesterol lowering mechanism, due to their role in promoting growth and proliferation of intestinal probiotic bacteria (Ziar *et al*., 2014).

The population and diversity of LAB and bifidobacteria present in feces of exclusively breast-fed (BF), formula-fed (FF) and combination-fed infants (CF) were investigated during early birth period. The impact of baby diet on diversity of LAB and bifidobacteria was studied using PCR-DGGE technique. In the meantime, quantitative analysis was performed to enumerate the LAB and bifidobacteria through FISH technique. Probiotic bacteria isolated from infant feces and breast milk were screened based on cholesterol-lowering property, and the cholesterol reducing mechanisms were then evaluated.

## Literature reviews

### 1. Human gastrointestinal microflora

The microbiota commensals play an important role in human health by acting as a barrier against pathogens and their invasion with a highly dynamic modality (Isolauri *et al*., 2004). These comprise over 1,000 different species (Fooks *et al*., 1999). It is discovered a variety of environmental conditions within the different parts of GI tract. The strong influences of colonization patterns are pH, peristalsis, redox potential, bacterial adhesion, mucin secretion, nutrient availability, diet and bacterial antagonism (wall *et al*., 2009). Under the strongly acidic condition of the stomach, a limited number of ingested bacteria reach the small intestine. Bacterial counts in the stomach and duodenum are usually less than $10^3$ per gram due to acidic pH. The number increase to $10^4$-$10^7$ per gram in the jejunum and ileum where rapid transit and the secretion of bile and pancreatic juice limit growth. The dominance of acid-tolerant included lactobacilli, streptococci and enterococci was found in the upper small intestine. The large intestine is the area most populated due to favorable conditions including slow gut transit times, nutrient availability and a favorable pH (Fooks *et al*., 1999). The population average $10^{10}$ -$10^{11}$ bacteria per gram and is dominated by Gram-positive bacteria such as *Lactobacillus, Streptococcus* and bifidobacteria. These bacteria can be recognized as beneficial, potential pathogenic and pathogenic bacteria (Westerbeek *et al*., 2006). The main groups of strict anaerobes bacteria in large intestine include *Bacteroides* sp., *Eubacterium* sp. and *Bifidobacterium* sp. Facultative aerobes such as *Enterobacter* sp. *Streptococcus* sp. and *Lactobacillus* sp. are also represent as subdominant flora. The pathogenic and opportunistic bacteria are also detected but in low numbers (Vaughan *et al*., 2000; Isolauri *et al*., 2004; Leahy *et al*., 2005; Westerbeek *et al*., 2006)

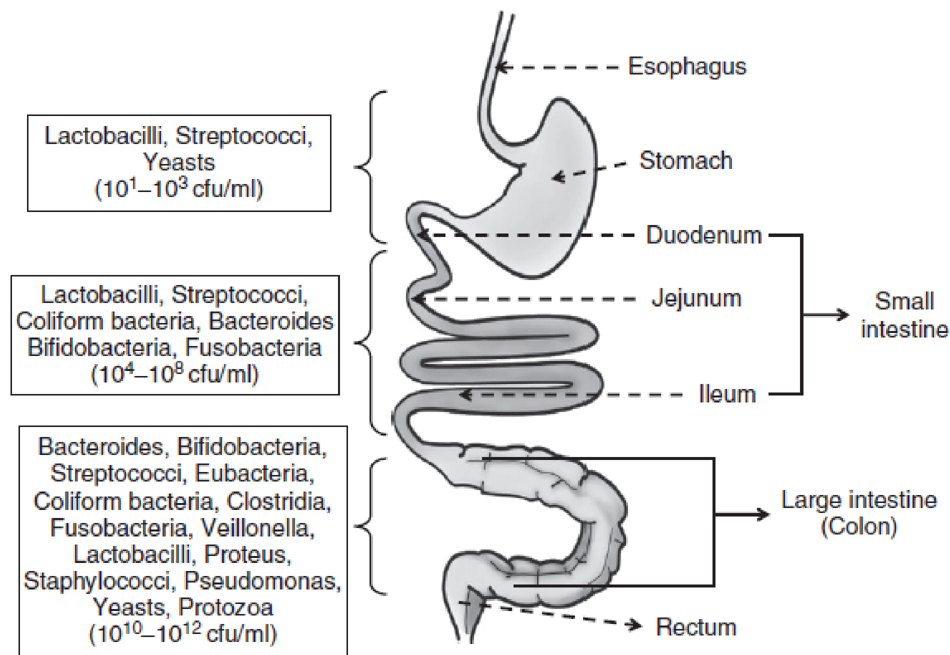


Figure 1. The microbial genera dominantly reside in the different parts of human gastrointestinal tract.

Source: Ariefdjohan *et al*. (2013)

**2. Development of gut microbiota in humans**

The gut remains sterile while the fetus is in the uterus but it becomes colonized immediately after birth. The most influential factors of gut microbiota are delivery mode and infant age. The GI tracts of natural born infants are colonized at first by fecal and vaginal bacteria derived from their mothers (Penders *et al*., 2006). The longer the birth process the more likely it is to find viable microbes in the infant's stomach and mouth. *Escherichia coli* and streptococci commonly colonize in infant gut immediately after birth. The influential factor of bacterial colonization in cesarean born infants is the environment. Infants born by cesarean do not come in contact with the maternal vaginal and fecal microorganisms and may be separated from the mother for a long period after birth. In this situation the environment becomes a crucial source of colonizing bacteria. These bacteria are mostly introduced from the environment of the hospital (Rutayisire *et*

*al*., 2016). In exclusive breast-fed infants, the numbers of *Bifidobacterium* sp. is a sharp increase, while the numbers of *E. coli* and *Streptococcus* sp. are decrease and *Clostrium* sp. low or absent. In exclusive formula fed infants, *Bacteroides* sp., *Clostridium* sp. and *Streptococcus* sp. are high prevalence, whilst *Bifidobacterium* sp. is present but not predominating. This strongly indicated that the diet can influence the ratio between the microbial species and strains of the intestinal microflora. In addition, the microflora in exclusive breast-fed infants becomes similar to exclusive formula-fed infants and *Clostridium* sp., *Streptococcus* sp. and *E. coli* were high level when exclusive breast-fed infants are provided with food supplements. After weaning, adult-type microorganism such as *Bacteroides*, *Prevotella*, *Ruminococcus*, *Clostridium* and *Veillonella* occurred in infants. In the second year of life, the intestinal microflora is similar to that of the adult (Gerritasen *et al*., 2011; Tanaka and Nakayama, 2017).

**3. Colonization and succession**

The colonization of the gut microbiota in GI tract occurs in four phases. (Orrhage and Nord, 1999). Phase 1, the initial occupy phase, occurs during 1-2 weeks. Phase 2, the transitional period, takes place during the third week after birth to the beginning of supplementary feeding. Phase 3 starts from the addition of solid food, especially at weaning period (e.g., when breast milk or formula milk is supplemented with solid foods). Phase 4, the period of convergence to adult microbiota patterns beginning after the completion of weaning (Mackie *et al*., 1999; Ariefdjohan *et al*., 2013). A consistent finding is that all infants are initially colonized by aerobic and facultative anaerobic such as *E. coli* and *Streptococcus* spp. These bacteria are thought to create a favorable environment for subsequent colonization by anaerobic bacteria, including *Bacteroides* spp., *Bifidobacterium* spp. and *Clostridium* spp. by day 4-7 (Stark and Lee, 1982; Mackie *et al*., 1999). In exclusively breast-fed infants, the reduction in number of *E. coli* and *Streptococcus* spp., as well as *Bacteroides* spp. and *Clostridium* spp., was observed and fecal *Bifidobacterium* spp. was dominant. Obviously, the intestinal microflora profile of exclusively formula fed infants is different, high numbers of *Bacteroides* spp.,

*Clostridium* spp. and *Streptoccus* spp. are present and *Bifidobacterium* spp. is no longer dominant in phase 2 (Ariefdjohan *et al*., 2013). The addition of solid foods to infants in phase 3 results in a major change in microbial succession. This change is more significant in exclusively breast-fed infants, the numbers of *Enterococcus* spp. and *Bacteroides* spp. increased, whereas enterobacteia and *Bifidobacterium* spp. remained constant, whereas exclusively formula-fed infants had smaller changes in the microflora during the conversion to solid foods, since they already harboured high numbers of aerobic bacteria and *Bacteriodes* spp. (Orrhage and Nord, 1999). Beginning of phase 4, bacterial succession continues until weaning is completed. *Bacteriodes* spp. and anaerobic gram-positive cocci continually increase in this phase. The levels of colonic *Bifidobacterium* spp. continue to remain high and populations of streptococci and *E. coli* decrease and by the second year of life the composition of intestinal microflora more closely resembles that of the adult. (Figure 2) (Stark and Lee, 1982; Edward and Parrett, 2002; Ariefdjohan *et al*., 2013).

**4. Factors affecting microbial colonization and succession**

Factors influencing gut microbial colonization and composition can be grouped into two main categories: external factors and internal factors. Microbial succession in the GI tract depends on numerous external and internal host-related factors. The external factors include delivery mode, diet composition, sanitation and antibiotic use. The internal factors are host physiological condition (e.g., stress, healthy human host and age) and GI tract environment (e.g., pH, substrate availability, redox potential, gut transit time, flow of enteric fluid and IgA secretion) (Mackie *et al*., 1999).

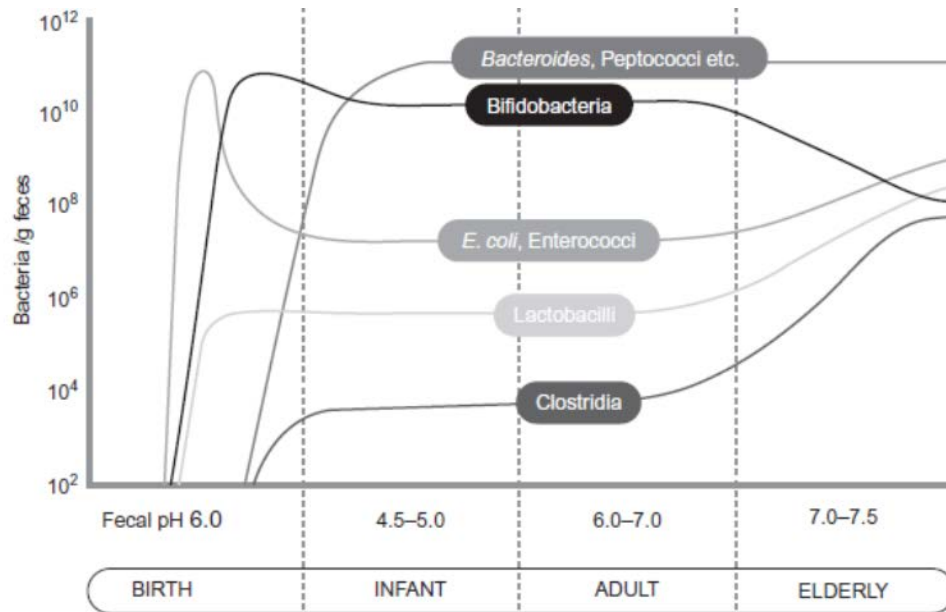


Figure 2. Bacterial succession of gut colonizers throughout human life time.
Source: Ariefdjohan *et al*. (2013)

### 4.1 Effect of the delivery mode on the gut microflora composition

During the birth process and immediately after birth, the infant is exposed by microbes from the mother and surrounding environment and microorganisms colonize the GI tract of infant resulting to the development of a dense complex microbiota. The initial microbial colonization patterns represent in the colon have an effect on several aspects of the infant health. (Le Huërou-Luron *et al*., 2010). The delivery mode has been revealed to have a strong influence on early gut colonization. Natural born infants allow direct contact with the fecal maternal microbiota (Makino *et al*., 2013). Conversely, the major sources of the microbiota for cesarean-born infants are found in the hospital environment and the mother's skin. Furthermore, *Bifidobacterium*, *Bacteroides*, and *E. coli* delay to colonize in cesarean-born infants. Moreover, natural born infants have higher numbers of *Bifidobacterium* compare to cesarean-born infants (Chen *et al*., 2007).

**4.2 Effect of infant feeding on the gut microflora composition**

The feeding type, either breast or formula milk, is strong influence in the infant gut microbiota development. Infant formulae are mainly based on cow's milk, supplemented with vegetable oil, minerals, and vitamins as a substitute for human milk. However, there is different of the composition between formula milk and exclusive breastfeeding such as oligosaccharides, antibodies, and bioactive proteins. Breastfeeding not only promotes the development of infant but also guides the development of the infant gut microbiota by preventing pathogen colonization and also by selecting bacteria. Interestingly, bifidobacteria and lactobacilli can use human milk oligosaccharide (HMO) as a growth substrate. During the first week of life the initial colonizers commonly found in both exclusively formula-fed and exclusively breast-fed infants are enterobacteria and *Streptococcus* spp. These reach their highest numbers by age 3.3 day on average. *Clostridium* spp. is always lower in exclusively breast-fed infants. The *Bifidobacterium* spp. and *Bacteroides* spp. appear 1 day later and then reach their highest numbers on days 5-6. Remarkably, *Bifidobacterium* spp. and *Bacteroides* spp. take a day longer to colonize as observed in the exclusively breast-fed infants (Mackie *et al*., 1999). Breast milk contains specific bifidobacterial growth factors which stimulate the growth of *Bifidobacteria* spp. (Kunz and Rudloff, 1993). Moreover, this genus is the most abundant in the infant gut of exclusively breast-fed infants and exclusively formula-fed infants (Magne *et al*., 2006; Boesten *et al*., 2011). However, exclusively breast-fed infants, viable count of fecal bifidobacteria was detected in larger numbers than the exclusively formula-fed one. *Bifidobacterium* spp. are more often isolated from exclusively breast-fed (84%) than the exclusively formula-fed infants (62%) for 1-week-old. In general, a small number of *Bifidobacterium* species are frequently found exclusively breast-fed infants including *B. longum, B. infantis*, *B.bifidum*, and *B. breve* (Rinne *et al*., 2005; Sakata *et al*., 2005). *Bifidobacterium* found in feces from formula-fed infants is more diverse which includes the aforementioned species and also *B. adolescentis* and *B. pseudocatenulatum* (Turroni *et al*., 2009). This is consistent with the concept that formula feeding initiates development of gut microbial profile to be more closely resemble to the

adult one (Mackie *et al*., 1999). Furthermore, lactobacilli show much lower population counts than, e.g., *Bacteroides* and bifidobacteria. The most common lactobacilli in breast-fed infants and formula-fed infants are *L. rhamnosus* and *L. gasseri*, which are later replace by *L. paracasei, L. plantarum, L. acidophilus* and *L. delbruckeii* (Ahrne *et al*., 2005). The differences in fecal microbiota between breast-fed and formula-fed infants may have decreased due to the development of improved infant formulae (Harmsen *et al*., 2000). The specific oligosaccharide supplementation is also reported to stimulate the growth of bifidobacteria and lactobacilli in the intestine resembling the effect of breast-feeding (Boehm *et al*., 2002).

## 5. Functions of intestinal microbiota

The human gut microbiota contributes to overall health. Disturbance of the ecological balance in gastro intestinal system may be dangerous to health. The human gut microbiota may possess a range of beneficial properties to the host by directing intestinal epithelial cell proliferation and differentiation, stimulate the immune system and produce digestive and protective enzymes. Moreover, these microbes are able to degrade certain food components in order to produce certain B vitamins. The main metabolic function of the colonic microflora is fermentation of polysaccharide (resistant starches, cellulose, hemicellulose, pectins and gum) and some oligosaccharide. The primary end-products of fermentation are short-chain fatty acids that are passively absorbed by the enterocytes (Holzapfel *et al*., 1998). Fermentation of different types of oligosaccharide is beneficial to the host as it provides additional energy in the form of short-chain fatty acids, butyric acid, acetic acid and proionic acid are the main short-chain fatty acids also involved in the salvage of energy (Leahy *et al*., 2005). Butyric acid is a main energy source for the intestinal epithelium and it is important in maintaining mucosal health in the colon (Isolauri *et al*., 2004). The gut microflora can often prevent attachment of pathogenic and opportunistic microorganisms into epithelial cells (Holzapfel *et al*., 1998).

**6. Microflora balance**

The microbiota inhabiting in the intestines have adapted to the hostile environment, including lysozyme, gastric and bile acids, and intestinal flow. The balance of the bacteria must be maintained for the intestine of function optimally. Moreover, this appears to be increasingly difficult as lifestyle changes. Several factors can shift the balance of the gut microbiota away from beneficial bacteria or health promoting bacteria such as *Lactobacillus* sp. and *Bifidobacterium* sp., and towards a predominance of potentially harmful or pathogenic bacteria, such as *Clostridium* sp., and *Bacteroides* sp. The predominance of these bacteria may pre-dispose toward a number of clinical disorders. These include cancer, inflammatory disease, and ulcerative colitis by making the host more susceptible to infections of enteropathogens like *Salmonella* sp., *Campylobacter* sp., *E. coli* and *Listeria* sp. (Fook *et al*., 1999).

**7. Probiotic**

There are many definitions about probiotic, a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance Fuller, 1989. This revised definition emphasizes the requirement of viability for probiotic and introduces the aspect of a beneficial effect on the host. Besides, these are viable mono or mixed culture of microorganisms which applied to animal or man, beneficially affects the host by improving the properties of the indigenous microflora and located in the human intestine, indigenous strains of lactobacilli and bifidobacteria. (Schrezenmeir and Vrese, 2001). Viable microbial food supplement which taken in the right dose beneficially influence the health of the host (Salminen and Isolauri, 2006). These may prove to be very effective (Fook *et al*., 1999). Selection criteria for probiotic microorganisms include:

**7.1 Probiotic strain must be safe (Generally regarded as safe; GRAS)**

Selected strains should be non-pathogenic and nontoxic. Generally, the LAB has good record in safety. The strains of microorganisms should be Generally Recognized as Safe (GRAS), for example *Lactobacillus* species, *Bifidobacterium* species and *Streptococcus* (*Enterococcus*) species, and should be following Qualified Presumptions of Safety (QPS) considering by the European Food Safety Authority (EFSA) (Holzapfel and Schillinger, 2002). Probiotics strains must be characterized at a minimum with the following tests:

- The genus, species and strain and its origin which will provide an initial indication of the presumed safety in relation to known probiotic and starter strains.
- Studies on the intrinsic properties of each specific strain such as resistance to gastric acidity, bile acid, digestive enzyme, adhesion and antimicrobial microbial activity against pathogenic bacteria as well as virulence factors.
- *In vivo* studies for substantiation of health effects in the target host.

(Holzapfel and Schillinger, 2002; Huy *et al*., 2013).

**7.2 Resistance to gastric condition**

The survival of probiotic during passage through the gastrointestinal tract is generally considered a key feature to preserve their expected health-promoting effects (Bezkorovainy, 2001). The secretion of gastric acid constitutes a primary defense mechanism against most ingested microorganisms. In LAB one of the most effective mechanisms for resistance in acid stress environment is $F_0F_1$-ATPase. *Lactobacillus* sp. and *Bifidobacterium* sp. show high tolerance to acid pH during 90 min incubation which is decreased after 2 h but individual strains vary considerably (Dunne *et al*., 2001). *L. acidophilus* can pass through the whole gut at high concentration. The strain was examined for resistance to pH 2-4 (Pan *et al*., 2009). In addition, different strains of *L. plantarum* were found to show a high tolerance to the consecutive exposure to hydrochloric acid (pH 2.0) (De Vries *et al*., 2006). *Lactobacillus* sp. proved more acid resistance than the *Bifidobacterium* sp. when exposed to the human gastric juice. The

viability of bifidobacteria at gastric juice pH values is considered to be generally low with the exception of *B. animalis* subsp. *lactis.* (Charteris *et al*., 1998; Takahashi *et al*., 2004). This strain is excellent survival when expose to human gastric juice (Gueimonde *et al*., 2004).

### 7.3 Bile acid resistance

When evaluating the potential of using LAB as effective probiotic it is generally considered necessary to evaluate their ability to resist the effects of bile acids (Lee and Salminen, 1995). Bile acids are synthesized in the liver from cholesterol and secreted from the gall bladder into the duodenum (Boyer, 2013). The conjugated bile can be transformed to deconjugation form. Both conjugated and deconjugated bile acids exhibit antibacterial activity, primarily through the dissolution of bacterial membranes (Begley *et al*., 2005). Deconjugated bile acid has decreased its solubility and diminished detergent activity and maybe less toxic to bacteria in the intestine than conjugated bile (Moser and Savage, 2001). Deconjugation is catalyzed by bile salt hydrolase (BSH) enzymes (EC 3.5.1.2.4), which hydrolyze the amide bond and liberate the glycine/taurine moiety from the steroid core (Begley *et al*., 2005). The BSH activity benefits the bacterium by enhancing its resistance to conjugated bile salts and increasing its survival in the gastrointestinal tract for colonization (Ridlon *et al*., 2006). *Lactobacillus* and *Bifidobacterium* strains isolated from the human ileum were tested against a bovine bile, porcine bile and human bile. The result observed for both the strain exhibited resistance to the bovine bile while the porcine bile proved significantly more inhibition to both of these bacterial groups (Dunne *et al.*, 2001).

### 7.4 Adherence to intestinal cell lines

The ability of attachment of bacterial strain to the intestinal mucosa is one of the main selection criteria for probiotic (Ouwehand *et al*., 1999). Adhesion to the intestinal mucosa may prevent the probiotic cells being washed out and therefore, enabling temporary colonization, immune modulation and competitive exclusions of pathogens. In

order to produce enzymes, lactic acids, vitamins and natural antibiotics, the probiotic strain must be adhere to the intestinal wall, colonize and multiply. Probiotic strain isolated from the human gut adheres and colonizes better than probiotic strain isolated from the animal origin (Walker and Duffy, 1998). Many factors involved in the adhesion of lactobacilli included surface component lipoteichoic acid (Granato *et al*., 1999), surface proteins (Chen *et al*., 2007), hydrophobic interaction (Van Tassell and Miller, 2011) and cell auto-aggregation ability (Kos *et al*., 2003). Many surface proteins of lactobacilli functioned as adhesion molecules included mucus adhesion promoting protein (MapA) in *L. reuteri* 104R (Buck *et al*., 2005), mucus- and mucin binding protein (Mmubp) in *L. fermentum* BSC87 (Miyoshi *et al*., 2006), and mucus-binding protein (Mub) in *L. acidophilus* (Macias-Rodriguez *et al*., 2009). Caco2 and HT-29 cell line, the two colonic adenocarcinomas are human intestinal epithelium derived, expressing structural and functional features of normal human enterocytes have been extensively used to screen putative probiotic (Chauviere *et al*., 1992). The ability to tolerate gastroenteric environment and the adhesive capacity to HT-29 cells among *Bifidobacterium* strains was different. Moreover, *B. breve* A04 had higher adhesive capability to HT-29 cells *in vitro* and average adhesive bacteria numbers reached 12.8 ± 0.9 for each HT-29 cell (Liu *et al*., 2007). Also, Tuomola and Salminen (1998) studied the adhesion of some probiotic and dairy *Lactobacillus* strains to Caco-2 cell cultures. *L. casei* was the most adhesive strain and *L. casei* var. *rhamnosus* was the least adhesive strain, approximately 14 and 3% of the added bacteria adhered to Caco-2 cell cultures, respectively.

### 7.5 Antimicrobial activity

LAB strains often produce antimicrobial substances with effect against gastric and intestinal pathogens and other microbes. LAB produced antimicrobial substances by fermentation carbohydrate. However, the fermentation depends on the oxidation of carbohydrates and related sub-products to generate end-products (Lindgren and Dobrogosz, 1990). The fermentation of hexoses by homofermemtative LAB is able to

convert available energy source almost completely into lactic acid via pyruvate (Reis *et al*., 2012). Heterofermentative LAB degrades hexoses and can lead to the generation of many other metabolites. These include acetate acid, acetoin, ethanol, carbon dioxide (Lindgren and Dobrogosz, 1990), together small amount of aromatic compounds such as diacetyl and acetaldehyde.

### 7.5.1. Organic acids

Organic acid levels and types of organic acids produced during the fermentation process depend on LAB species or strains, culture composition and growth conditions (Ammor *et al*., 2006). It has been proposed that the low external pH causes acidification of the cell cytoplasm, while the undissociated acid acts by collapsing the electrochemical proton gradient, or by altering the cell membrane permeability which of substrate transport systems of susceptible bacteria, leading to bacteriostasis and eventual death (Ammor *et al*., 2006, Soomro *et al*., 2002; Tome *et al*., 2006). Recently, Leon *et al*. (2012) published a very interesting study about the inhibitory activity of lactic and acetic acid for antifungal activity against different strains of *Aspergillus flavus*. De Keersmaecker *et al*. (2006) demonstrated the spent culture supernatant (SCS) of the probiotic *L. rhamnosus* GG under the growth conditions produced lactic acid to exert antibacterial activity against *Salmonella* Typhimurium.

### 7.5.2. Hydrogen peroxide

The production of $H_2O_2$ by LAB can prevent the growth of foodborne pathogens and can also be beneficial in food preservation. $H_2O_2$ is generated by some micro-organisms growing aerobically and usually generated by lactobacilli present in a healthy vagina, but is mostly absent in women with bacterial vaginosis (Batdorj *et al.*, 2007; Dover *et al*., 2008). Also $H_2O_2$ could react with other cellular and milieu components to form additional inhibitory substances (Lindgren and Dobrogosz, 1990). The amount of $H_2O_2$ in the culture supernatant increased during bacterial growth and reached a maximum (5.12 mmol/L) at the early stationary phase under aerated conditions

(agitated cultures) but was not detected in the culture performed without agitation (Batdorj *et al.*, 2007).

### 7.5.3. Bacteriocins

Bacteriocins are classified as ribosomally synthesized antimicrobial peptides, and they are produced by different groups of bacteria (Cotter *et al*., 2005). Bacteriocins production could be considered as advantageous to the producer as, in sufficient amounts; these peptides can kill or inhibit bacteria coming for the same ecological niche or the same nutrient pool (Deegan *et al.*, 2006). It can inhibit the growth of Gram-positive pathogenic and spoilage bacteria as well as yeasts, besides, it has been reported that bacteriocins also inhibit the growth of some Gram-negative species (Topisirovic *et al.*, 2006). Most bacteriocin-producing LAB have been isolated from fermented foodstuffs but bacteriocin-producing human strains of *Lactobacillus* or *Bifidobacterium* have been isolated from the human intestine, stool or vaginal tract (Millette *et al.*, 2007; Toure *et al.*, 2003). *L. salivarius* UCC118, a recently sequenced and genetically tractable probiotic strain of human origin, produces a bacteriocin *in vivo* that can significantly protect mice against infection with the invasive foodborne pathogen *Listeria monocytogenes* (Corr *et al.*, 2007).

### 7.5.4. Diacetyl and Acetaldehyde

Diacetyl or 2, 3-butanedione may be synthesized by either homolactic or heterolactic pathways of sugar metabolism as well as by utilization of citric acid (Hugenholtz, 1993). Diacetyl can be further transformed into acetoin as well as acetaldehyde (2, 3-butanediol). Its role involved in antimicrobial activity against various food-borne pathogens and spoilage microorganisms. These included Gram-negative bacteria, yeasts and molds. The antimicrobial effect of diacetyl may result from interfere with arginine utilization by reacting with the arginine-binding protein of Gram-negative bacteria (Jay, 1982). Motlagh *et al.* (1991) demonstrated that diacetyl (344 ppm) against *E.coli*, and *Salmonella*, but not *Listeria monocytogenes*. Similarly, acetaldehyde also

plays a role in controlling the growth of contaminants together with other antimicrobial metabolites of LAB.

**8. Cholesterol-lowering mechanism of probiotic**

Cholesterol is a soft waxy substance that is a natural component of certain fats in the bloodstream and in all the cells of the body. Although, cholesterol is an essential part of a healthy body, elevated blood cholesterol is a well known major risk factor for cardiovascular disease which is the leading cause of one-third of all deaths worldwide (Law *et al*., 1994; Ma, 2006; Ajmal and Ahmed, 2009; Labarthe and Dunbar, 2012). Recent modalities for lowering blood cholesterol levels involve dietary management, behavior modification, regular exercise and drug therapy (Kumar *et al*., 2012). Phamacological agents that effectively reduce cholesterol levels are available for the treatment of high cholesterol; however, they have been associated with many adverse effects that limit treatment compliance as well as quality of life (Bliznakov, 2002; Gotto and Moon, 2012). Therefore, there is growing interest in the preventing and lowering blood cholesterol level by practicing dietary control or using probiotic such as *Lactobacillus* spp., *Bifidobacterium* spp. and *Enterococcus* spp. has emerged recently (Pereira and Gibson, 2002; Guo *et al*., 2012; Helim *et al*., 2016). LAB and bifidobacteria have a major role to play in the cholesterol lowering mechanism (Table 1) base on relevant characteristics including enzymatic deconjugation of bile acid by bile salt hydrolase (BSH), co-precipitation of cholesterol with free bile acids, binding cholesterol to surface, or assimilation of cholesterol into the cellular membrane or cytoplasm (Ahn *et al*., 2003; Lye *et al*., 2010; Tomara-Duchesneau *et al*., 2014).

Table 1. Summary of major findings of probiotic bacteria mediated cholesterol reduction.

| S. No. | Probiotic organism | Experimental system | Major findings |
|---|---|---|---|
| 1 | Unknown (fermented milk) | Maasai tribesmen in Africa | Low cholesterol |
| 2 | Unknown (Yogurt) | Human subjects | Reduced cholesterol |
| 3 | *Lactobacillus acidophilus* | Culture medium | Cholesterol removal |
| 4 | *Bifidobacterium* | Culture medium | Removal of Cholesterol |
| 5 | *Lactobacillus acidophilus* | Culture medium | Cholesterol assimilation |
| 6 | Probiotic fermented milk | Rats | Cholesterol reducing efficacy |
| 7 | *Lactobacillus reuteri* | Mice | Reduced blood cholesterol<br>Decrease triglycerides |
| 8 | *Bifidobacterium* | Rats, Human | Reduced cholesterol<br>Decreased triglyceride<br>Decrease LDL<br>Increased HDL |
| 9 | Yoghurt containing *B. lactis* or *B. longum* | Rats | Reduced cholesterol<br>Decreased triglyceride<br>Decrease LDL<br>Increased HDL |
| 10 | *L. plantarum* | Culture medium | Cholesterol assimilation |
| 11 | *L. bulgaricus* and *L. acidophilus* | Human | Decreased cholesterol |
| 12 | *Lactobacillus sporogenes* | Human | Decreased cholesterol<br>Deduced LDL-cholesterol |
| 13 | *L. acidophilus* | Human | Decreased cholesterol |
| 14 | *Enterococcus faecium* | Human | Decreased cholesterol levels<br>Decreased triglyceride<br>Decrease LDL<br>Increased HDL |
| 15 | Microencapsulated bile salt hydrolase active *Lactobacillus reuteri* NCIMB 30242 | Human | Reduced LDL-cholesterol<br>Decreased total cholesterol<br>Decreased apoB-100<br>Decrease non-HDL-cholesterol |

**8.1 Enzymatic deconjugation of bile acid by bile salt hydrolase (BSH)**

Probiotics such as *L. acidophilus*, *L. plantarum*, *B. bifidum* and *Ent. faecium* were found to excrete BSH enzyme (cholylglycine hydrolase; EC3.5.1.24), that catalyzes the hydrolysis of glycine-and taurine-conjugated bile salts into amino acid residues and termed unconjugated or deconjugated bile acids in the enterohepatic circulation (Moser and Savage, 2001; Wijaya *et al*., 2002; Liong and Shah, 2005; Parvez *et al*., 2006). Cholesterol is the precursor of primary bile salts that are synthesized in the liver and are stored and concentrated as conjugated bile salts in the gall bladder. Conjugated bile salts are released into the small intestine for absorption of dietary fat, hydrophobic vitamins and other fat-soluble compounds (Liong and Shah, 2005; Begley *et al*., 2006). Once deconjugated, bile salts are less soluble and less efficiently absorbed by the intestines than conjugated bile salts, resulting in their elimination in the feces. Cholesterol is required for the synthesis of new bile acids in a homeostatic response, resulting in lowering of serum cholesterol (Ooi and Liong, 2010).

**8.2 Co-precipitation of cholesterol with free bile acids**

The removal of cholesterol was due to the disruption of the cholesterol micelles caused by cholesterol is co-precipitate with deconjugated bile salts at acidic condition (pH < 5.5) during growth (Klaver and Van der Meer, 1993; Tahri *et al*., 1996; Liong and Shah, 2005). Although, physiological pH of intestine is neutral to slightly alkaline that is unsuitable for cholesterol co-precipitation mechanism. However, short-chain fatty acid, the end product of fermentation of dietary fibers by intestinal microbiota is able to transfer the microenvironment into acidic condition, ensuring the occurrence of co-precipitation (Begley *et al*., 2006).

### 8.3 Assimilation of cholesterol into the cellular membrane or cytoplasm

Cholesterol was also removed by bacterial cell by up-take or assimilation into the cellular membranes during growth thus making it unavailable for absorption from the intestines into the blood (Walker and Gilliland, 1993; Liong and Shah, 2005). Several strains of *Lactobacillus*, *Bifidobacterium* and *Enterococcus* were able to remove cholesterol from the medium during growth (Pereira and Gibson, 2002; Sirilun *et al*., 2010; Guo *et al*., 2016). Moreover, cholesterol assimilation was associated with the presence of bile salts and cholesterol removal from the medium increased with increasing concentration of bile salt (Lye *et al*., 2010).

### 8.4 Binding cholesterol to bacterial cell surface

Cholesterol binding to cell surfaces is the mechanism by which several strains of probiotic are able to remove cholesterol (Kumar *et al*., 2012; Pavlovic *et al*., 2012). Growing cells were compared to those that were non-growing (live but suspened in phosphate buffer) and dead (head-killed), although greater cholesterol removal by growing cells than non-growing cells and dead cells. However, the non-growing and heat-killed cells are able to remove cholesterol by binding on the cellular surface (Kimoto *et al*., 2002; Lye *et al*., 2010).

## 9. Fluorescent in situ hybridization (FISH)

Fluorescent in situ hybridization (FISH) is a molecular technique that uses for detection of specific bacterial groups in mixed populations without prior cultivation (Langendijk *et al*., 1995). The technique of FISH is based on the same principle as any DNA hybridization method which uses the ability of single-stranded DNA to hybridize to complementary DNA. The oligonucleotide probe of genus-specific 16S rDNA is tagged with a fluorescent dye that when observed under a fluorescent microscope with the specific wavelength of light will fluoresce (Vaughan *et al*., 2005). This method can be used to detect bacteria with different phylogenetic levels which are depended on the probe chosen (Raskin *et al*., 1994). The FISH technique has been widely used to quantify

bifidobacteria or other specific groups from fecal microflora (Franks *et al*., 1998; Mangin *et al*., 2002). The first 16S rRNA oligonucleotide probe which performed human fecal bifidobacteria was Bif164. Dinoto *et al*. (2006) successfully applied FISH to evaluate the effect of raffinose administration on the modulation of rat cecal microbiota. Also, He *et al*. (2007) investigated the effects of supplementation of *B. longum* in capsules and a yogurt containing *B. animalis* on the fecal bifidobacterial population. The other nucleotide probes were designed to be specific for bacterial groups in fecal samples. These include Bac 303, Lab 158, Chis 150, and Eub 338 specific for *Bifidobacterium*, *Bacteroides*, *Lactobacillus/Enterococcus* spp., *Clostridium histolyticum* group, and *Eubacterium group*, respectively (Harmsen *et al*., 1999; Franks *et al*., 1998; Mangin *et al*., 2002).

**10. Denaturing Gradient Gel Electrophoresis (DGGE)**

This method was originally invented to detect point mutations in healthy related studies. The separation power of DGGE relies on the fact that single strand (ss) DNA migrates more slowly than double strand (ds) DNA during electrophoresis. This is due to an increased interaction of the branched structure of the single-strand moiety with the gel matrix via the individual nucleotides hanging freely from the sugar phosphate backbone of the helix and the becoming entangled in the gel matrix. By contrast, dsDNA more than compensates for its double weight by gels stacking of the nucleotides within the helix structure. Since the nucleotides are interacting with each other via hydrogen bonding, the dsDNA will pass much faster through a gel matrix than a ssDNA molecule. If consider two almost identical PCR products (e.g. alleles), that differ by only one nucleotide within a low melting domain of the amplified sequence, these products will have different melting temperature. A DGGE gel typically consists of a polyacrylamide gel across which an electric charge is passed. The gel is formed so that there is an increasing denaturing gradient formed by the addition of chemical denaturants (usually formamide and urea). When a dsDNA PCR product migrates through a DGGE gel, it is therefore subject to increasing denaturation. The separation of DGGE is based on the

electrophoretic mobility of a partially melted DNA molecule in polyacrylamide gels (Muyzer *et al*., 1993). Depending on the composition of the sequence, in particular in terms of its % GC composition, the molecule will start to denature and the two strands separate. Thus migration of the molecule is retarded due to the growing interaction of the charged nucleotide with the charged gel matrix. However, the rate of migration will increase again once the two strands are completely separated. Therefore in DGGE, complete strand separation is prevented by the presence of a high melting domain in the DNA molecule, which is usually artificially created at one end of the molecule by the incorporation of a GC clamp. This is accomplished via the use of the oligonucleotide primer for PCR amplification that includes a 5' tail consisting of a sequence of 40 guanine and cytosine residues. The GC clamp is usually positioned adjacent to the highest melting domain of the amplicon to efficiently force the progress of melting in only one direction on the molecule, thus avoiding the existence of different molecule formations that may cause either a smear or more than one band per sequence (Daims *et al*., 2005). The GC clamp on a primer (figure 3) is an essential requirement for successful DGGE analysis. Whilst GC-clamped primers consisting of an extended primer together with a GC-rich region at the 5' end of the primer can be readily purchased from commercial suppliers, the synthesis of such oligonucleotides (typically 60 bp) is not trivia. Hence, primer should be purchased that are purified using the best available purification method; shortened GC clamps may spoil results by an extended ssDNA smear. Muyzer *et al*. (1993) who first applied DGGE performance of his initially published GC clamp. When designing a GC clamp, repetitive sequence should be avoided to ensure proper annealing of the GC clamp during the PCR. It is likely that the GC clamp may hamper primer annealing during PCR and reduce amplification efficiency. Therefore, it is advisable to add GC clamps to standard primers that have already been shown to have high PCR efficiency.

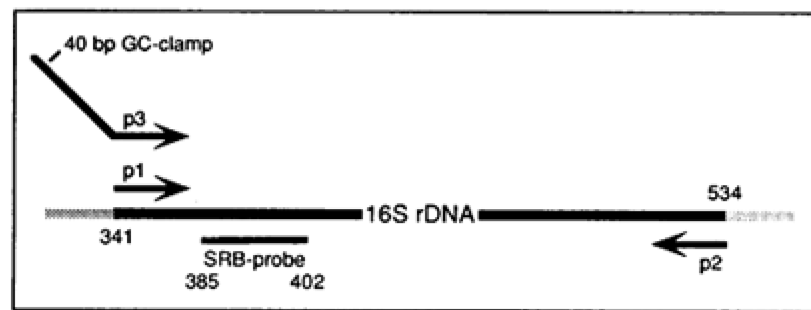


Figure 3. Application of GC clamps in DGGE analysis. Tailed primers are used to attach the GC clamp to amplicons, to prevent formation of ssDNA during DGGE analysis.

Source: Muyzer *et al*. (1993)

**Objectives**

1. To examine infant-related factors influenced on the population and diversity of fecal beneficial bacteria.
2. To investigate the relationship between feeding type and beneficial bacteria development of infant.
3. To screen LAB and bifidobacteria isolated from infant feces and breast milk based on cholesterol-lowering ability.
4. To investigate cholesterol lowering mechanisms of probiotic strains isolated from infant feces and breast milk.

# CHAPTER 2

# FACTORS INFLUENCING THE POPULATION AND DIVERSITY OF BIFIDOBACTERIA IN THAI HEALTHY NEWBORNS

## 2.1 Abstract

Gut colonization of bifidobacteria in early infancy is essential for the well-being of the infant. Several factors have involved in bifidobacterial colonization, such as delivery mode, infant age, antibiotic treatment and feeding type. Factorial experimental design was applied to evaluate the factors including delivery modes (natural born and Cesarean born), feeding types (breast milk, formula milk and combination milk) and ages of infant (1 week, 1 month, 2 months, 3 months, 4 months and 5 months) on the bifidobacterial population and composition of infant feces. Among 6 healthy newborn infants, infant age and feeding type were the most influential factor for the population of bifidobacteria, clostridia, bacteroides and eubacteria, whereas feeding type and delivery mode were the main factors influencing total bacteria population. The amount of bifidobacteria rapidly increased from first week to 2 months of infant age and stable then after 2 months in all of infants. Moreover, the amount of clostridia, bacteriodes and eubacteria significantly increased from the first week up to 5 months of ages in all of infants. Feeding type was also main factor influencing on population of bifidobacteria, clostridia, bacteroides, eubacteria and total bacteria. The exclusive breast-feeding greatly supported the bifidobacterial growth. The level of bifidobacteria in both naturally born, exclusive breast fed infant (BF1-D) and Cesarean born, exclusive breast fed infant (BF2-E) was significantly higher than other infants. However, the highest level of bifidobacteria was found in naturally born, exclusive breast fed infant (BF1-D) from first week to 5 months of ages (8.79 to 9.83 log CFU/g feces). In addition, the amount of bifidobacteria in naturally born, formula fed infant (FF1-F) greatly increase (8.82 to 9.45 log CFU/g feces) when received the exclusive formula milk supplemented with *B. lactis* at 2 months to 5 months of age. Using antibiotic (amoxicillin) in Cesarean born, combination fed infant (CF2-C) was significantly decreased the amount of bifidobacteria

(9.21 to 8.77 log CFU/g feces) at 3 months of age. Moreover, formula milk seemed to enhance the growth of clostridia and bacteroides. The highest level of the clostridia was found in Cesarean born, exclusively formula fed infant (FF1-F) (9.22 log CFU/g feces) when received exclusive formula milk supplemented with FOS and GOS at 2 months of age. However, the amount of clostridia was significantly decreased when received the exclusive formula milk supplemented with *B. lactis*. Conversely, low level of clostridia was found in both naturally born, combination fed infant (CF1-A) and naturally born, exclusively breast fed infants (BF1-D). In addition, the amount of bacteroides was significantly increased (8.59 to 9.19 log CFU/g feces) in Cesarean born, formula fed infant (FF2-H) at the first week to 5 months of age. The amount of total bacteria in naturally born, exclusively breast fed infant (BF1-D) and naturally born, exclusively formula fed infant (FF1-F) was significantly higher than another infants.

PCR-DGGE analysis demonstrated that there was change in the bifidobacterial community composition associated with feeding type and delivery mode. The high numbers of bifidobacteria band were observed in both naturally born, exclusively breast fed infant (BF1-D) and naturally born, combination of breast and formula fed infants (CF1-A). However, the highest number of bifidobacterial species was observed in both naturally born, exclusively formula fed infant (FF1-F) and naturally born, combination of breast and formula fed infants (CF1-A). Several species of bifidobacteria including *Bifidobacterium adolescentis, Bifidobacterium* sp. *Bifidobacterium longum, Bifidobacterium catenulatum* and *Bifidobacterium breve* were found persistently from the first week to 5 months of ages in naturally born, both of breast fed (BF1-D) and combination fed infants (CF1-A). The most common bifidobacterial species found in healthy infants were *Bifidobacterium* sp. and *B. longum.*

**2.2 Introduction**

The gut microiota plays an important role for host health, especially as the early establishment of gut microbiota in a newborn infant is thought to be important for the development of the immune system (Quigley *et al*., 2013). The gastrointestinal tract of a healthy fetus is sterile, and it is rapidly colonized with bacteria after birth, early colonization begins with facultative anaerobes, such as enterobacteria, coliforms and lactobacilli and continues with anaerobic genera such as bacteroides, clostridia and bifidobacteria. (Bezirtzoglou, 1997; Pender *et al*., 2006). The critical stage of gut colonization occurs within the first few weeks after birth and is influenced by a variety of factors; including gestational age, delivery mode, infant age, hygiene, antibiotic treatment, geographical zone and feeding type (Thompson-Chagoyan *et al*., 2007; Marques *et al*., 2015). In early life, one of the first major factors of the gut microbiota is the mode of delivery. Natural born allows direct contact with the fecal maternal microbiota (Makino *et al*., 2013). Conversely, the major sources of the microbiota for cesarean-born infants are found in the hospital environment and the mother's skin. Furthermore, *Bifidobacterium*, *Bacteroides*, and *E. coli* delay to colonize in cesarean-born infants. Moreover, natural born infants have higher numbers of *Bifidobacterium* compare to cesarean-born infants (Chen *et al*., 2007). Moreover, microbial colonization is dependent upon age of infant, the first days and weeks of age represent a crucial window of opportunity for shaping the development of GI tract (Houghteling and Walker, 2015). In addition, the gut microbiota is less stable than later in childhood and there are changes in the phylogenetic diversity in the first months of age (Nuriel-Ohayon *et al*., 2016). Another strong influence in the formation of the intestinal microbiota in infancy is the type of feeding. Breast feeding and formula feeding are considered to be key factors that provide differential colonization opportunities and thus composition of the neonatal gut microbiota (Pender *et al*., 2006). Breast-fed infants have a gut microbiota that is more dominated by bifidobacteria, whereas in formula-fed infants, coliforms, bacteriodes and enterococci predominate (Azad *et al*., 2013; Tannock *et al*., 2013). Colonization with bifidobacteria is considered the most important in supporting health beneficial properties,

including the prevention of diarrhea and intestinal infections, human pathogen growth inhibition (Servin, 2004) and immunostimulation (Plaza-Diaz *et al*., 2014). Bifidobacteria appear after birth and within a week reaching the dominant bacterial group in healthy infants (Euler *et al*., 2005). Species that are frequently isolated are *Bifidobacterium infantis Bifidobacterium breve*, *Bifidobacterium longum*, *Bifidobacterium bifidum*, *Bifidobacterium dentium Bifidobacterium adolescentis*, *Bifidobacterium pseudocatenulatum* (Sakata *et al*., 2005; Aires *et al*., 2011). The aim in this study determined the impact of delivery mode, feeding type and infant age on the population and diversity of fecal beneficial bacteria.

## 2.3 Materials and Methods

### 2.3.1. Subjects and infant fecal samples preparation

The protocol of this study was reviewed and approved by the ethics committee of Faculty of Medicine, Prince of Songkla University (EC Number: 55-244-19-2-3). This study was carried out with 6 healthy newborn infants (Table 2). All infants were born at 37 to 42 weeks of gestation with no evidence of disease at the third day of life and no antibiotic treatment. All infants showed no sign of any health problem from birth until to the end of this study. Fecal samples were collected from the infants at ages 1 week, 1 month, 2 months, 3 months, 4 months and 5 months. The sample collected in a sterile plastic container was then immediately cooled and strictly maintained at 4 $^{o}$C throughout transportation period (not longer than 1 h). All fecal samples were kept frozen at -20 $^{o}$C.

### 2.3.2. The factorial experimental design and data analysis

The factorial experimental design was used statistically analyzed the following three factors: infant ages (A), feeding types (B) and delivery mode (C). The levels of the factors studied were shown in Table 3. The levels of the factors studied included first week, 1 months, 2 months, 3 months, 4 months and 5months as infant age; exclusive breast milk, exclusive formula milk and combination between breast and formula milk as

feeding types; natural born and Cesarean born as delivery mode. The experimental results were analyzed to determine the main effects of the factors independently. The analysis of variance technique (ANOVA) was then applied to determine which factors were statistically significant. All calculations were performed using Minitab software (trial version 15, Minitab Inc., USA).

### 2.3.3 Bifidobacteria and the dominant fecal microflora in infant feces using fluorescent *in situ* hybridization (FISH) technique

Homogenized infant fecal sample (375µl) were added to 1.125 ml of 4% (w/v) filter-sterilized paraformaldehyde solution (Acros, New Jersey, USA) and left overnight at 4$^{o}$C. The mixture was thereafter centrifuged at 8,100×g for 5 min at 4$^{o}$C. The fixed cells were washed twice with cold filter sterilized phosphate-buffered saline (PBS 0.1 M, pH 7.2), and then resuspended in 300 µl of PBS/99% ethanol (1:1w/v) mixture and stored at -20 $^{\circ}$C until further analysis, which was no longer than 3 months (Uraipan *et al*., 2014). FISH technique was performed as described by Hongpattatakere *et al.* (2012). The genus-specific 16S rRNA target oligonucleotide probe (Sigma, USA) labeled with the fluorescent dye Cy3 used was Bif 164 (5-CATCCGGCATTACCACCC-3) specific for *Bifidobacterium*, Bac 303 (5-CCAATGTGGGGGACCTT-3) specific for *Bacteriodes*, Chis 150 (5-TTATGCGGTATTAATCT(C/T)CCTTT-3) specific for *Clostridium*, Eub 338 (5-GCTGCCTCCCGTAGGAGT-3) specific for *Eubacterium* and nucleotide target 4',6-diamidino-2-phenylindole (DAPI) dye (Sigma, St. Louis, MO, USA) was used for total bacterial counts. Cell were counted using fluorescence microscope (Nikon Eclipse 80i, USA) with a minimum of 15 fields, (10-100 cells/field). The numbers of bifidoacteria and dominant fecal microflora present in each sample were determined using the following equation:

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

Where DF, ACC, $DE_{sample}$, 7079.68 and 50 represent the dilution factor (300/375=0.8); the average cell count drawn from 15 microscopic fields of view. The dilution of the sample used with a particular probe (e.g. 10× for Bif 164 count); the area

of the well divided by the area of the microscopic field; and the conversion factor taking the number based on milliliter of sample (Ogue-Bon *et al*., 2010).

### 2.3.4 Bifidobacteria diversity presents in infant feces using denaturing gradient gel electrophoresis (DGGE) technique.

#### 2.3.4.1. Extraction of genomic DNA from fecal samples

Infant fecal sample 1 g was washed in 1 ml with PBS then was centrifuged each preparation at 8,100×g for 5 min in order to reduce the PCR inhibitors. The pellet of fecal sample were resuspended in 100 µl of distilled water, serially diluted in 100 µl of 1% Triton X-100 (Fluka, Seelze, Germany), heated at 100°C for 5 min, and immediately cooled in ice water (Wang *et al*., 1996). The samples were resuspended in 100 µl of lysis buffer (6.7% sucrose, 50 mM TrisHCl (pH 8.0), 10mM EDTA, 20 mg of lysozyme/ml (Invitrogen, CA, USA), 1,000 U of mutanolysin/ml (Sigma-Aldrich, Steinheim, Germany), 100 mg of RNaseA/ml) (Amresco, Ohio, USA) according to Walter *et al.* (2001).

#### 2.3.4.2 Nested PCR-DGGE analysis

In order to increase sensitivity and to facilitate DGGE analysis, a nested PCR was performed to amplify target DNA. The first round PCR was performed with Im3-r (5' CGGGTGCTICCCACTTTCATG) and Im26-f (5' GATTCTGGCTCAGGA TGAACG) as specific primers (Balleste and Blanch, 2011). The PCR volumes of 50 µl contained 25 µl red dye PCR master mix (GeNei, Bangalore, India), 5 µL of each primer (2µM), 1 µl of genomic DNA and 14 µl of sterile Milli-Q water (Omnipur, Gibbstown, NJ, USA). The first round PCR products were used as templates in the next round of the amplification. The variable V2-V4 region (520 bp) of 16S rDNA of bifidobacteria was targeted using Bif164-f (5'-GGGTGGTAATGCCGGATG-3') and Bif 662-GC-r (GC-clamp 5'CGCCCGCCGCGCGGCGGGCGGGGCGGGGGCACGGGGGCCACCG TTACACCGGGAA-3') (Faveier *et al*., 2002; Balleste and Blanch, 2011). DGGE analysis of PCR amplicons were carried out on the DGGE system (Cleaver Scientific,

York, UK) under the condition described previously (Uraipan *et al*., 2014). The linear denaturing gradients of urea and formamide use for separation of amplicons from bifidobacteria were 35-60%. A 100% denaturing solution contained 40% (v/v) formamide (Amresco, Ohio, USA) and 7 M urea (Acros, New Jersey, USA). The electrophoresis was conducted with a constant voltage of 85 V at 60$^{o}$C for about 16 h (Uraipan *et al*., 2014). After electrophoresis, the gels were incubated for 30 min with 1× SYBR® Gold (Invitrogen, Grand Island, NY, USA), allowing digital capturing of the DGGE band profiles under UV light transillumination (Alpha innotech corporation, San Leandro, USA). The bifidobacteria diversity was evaluated by the number of bands.

The DGGE bands were cut under Ultra Bright LED Transilluminator (Gellex, Hsinchu City, Taiwan). Each individual DNA band was extracted by incubating in 100 µl of 1×TE buffer and overnight at 4 ℃. The obtained DNA was subsequently re-amplified by PCR using Bif164-f and Bif 662-r primers without GC clamp, before its nucleotide sequence was determined at Ward Medic Ltd., Part. (1st Base Distributor, Thailand). Closest matches for partial 16S rRNA gene sequences were identified by basic local alignment search tool (BLAST) with the nucleotide collection (nr/nt) database in the BLAST search program available at http://www.ncbi.nlm.nih.gov/.

### 2.3.5 Statistical analysis

All determinations were performed in triplicate. The data were subjected to analysis of variance (ANOVA) and significant differences among mean values were determined by SPSS 17.0. The values were expressed as means ± SD. Statistical significance was evaluated using Duncan's Multiple Range Test. Statistical significance was accepted at $P < 0.05$

Table 2. The condition of infant involved in this study

| **Infant codes** | **Gender** | **Delivery modes** | **Feeding types** | **Brand of milk powder** | **Duration of formula milk feeding** | **Antibiotic use** | **Age at start to introduce solid food** | **Remarks** |
|---|---|---|---|---|---|---|---|---|
| CF1-A | Male | Natural born | Combination milk | Hi-Q one plus brand (with FOS and GOS) | 1 week-5 months of infant age | No | 4 months (porridge, pumpkin and banana) | - |
| CF2-C | Male | Cesarean born | Combination milk | Enfalac brand (with inulin and GOS) | 1 week-5 months of infant age | Yes | 3 months (pumpkin, honey and carrot juice) | He had exanthematous fever and was treated with green medicine (yaa kieow) and amoxicillin 125 mg, half of tea spoon for 3 times/day for 1 week at 3 months of age |
| BF1-D | Female | Natural born | Breast milk | - | - | No | 4 months (banana) | - |
| BF2-E | Male | Cesarean born | Breast milk | - | - | No | 4 months (porridge and banana) | - |
| FF1-F | Male | Natural born | Formula milk | Hi-Q one plus brand (with FOS and GOS) Nan HA1 brand (hypoallergenic formula with *B. lactis* 0.00392%) | Hi-Q one plus brand (1 week-2 months) of infant age and Nan HA1 brand (2 months-5 months) of infant age | No | 4 months (porridge, banana and orange juice) | - |
| FF2-H | Male | Cesarean born | Formula milk | Enfalac brand (with inulin and GOS) | 1 week-5 months of infant age | No | 4 months (banana, pumpkin and boiled egg york) | - |

Table 3. Factors and their levels in the factorial experimental design for determination of the determinant on the populationof bifidobacteria and dominant fecal microflora.

| Factors | Level 1 | Level 2 | Level 3 | Level 4 | Level 5 | Level 6 |
|---|---|---|---|---|---|---|
| A: Infant ages | 1 week | 1 month | 2 month | 3 month | 4 month | 5 month |
| B: Feeding types | Breast milk | Formula milk | Combination milk | | | |
| C: Delivery modes | Natural born | Cesarean born | | | | |

## 2.4 Results

### 2.4.1 The condition of each infant in this study

Six full term Thai healthy infants (Table 2) (one female: naturally born, breast fed infant (BF1-D) and five males: naturally born, combination fed infant (CF1-A), Cesarean born, combination fed infant (CF2-C), Cesarean born, breast fed infant (BF2-E), naturally born, formula fed infant (FF1-F) and Cesarean born, formula fed infant FF1-H) have participated to the study. After birth, BF1-D and BF2-E infants received exclusive breast milk up to 5 months. They were then fed with solid foods (banana and porridge) at 4 month of age. CF1-A infant received breast milk and formula milk supplemented with fructo-oligosaccharide (FOS) and galacto-oligosaccharide (GOS) (Hi-Q one plus brand) and was fed with porridge, pumpkin and banana at 4 months of age. CF1-C infant received breast milk and formula milk supplemented with inulin and GOS (Enfalac brand). He was treated with amoxicillin and green medicine (Yaa kieow) for 7 days at 3 months of age due to exanthematous fever. In addition, he was received the pumpkin juice, honey and carrot juice as a solid food at 3 months of age. FF1-F infant was received exclusive formula milk supplemented with FOS and GOS (Hi-Q one plus brand) for 2 months of age, thereafter the formula milk was changed to hypoallergenic formula milk supplemented with *Bifidobacterium lactis* (Nan HA1 brand) until 5 months of age and received porridge, banana and orange juice at 4 months of age. FF2-H infant received

the exclusive formula milk supplemented with inulin and GOS (Enfalac brand) for 5 months of age and received banana, pumpkin juice and boiled egg york at 4 months of age.

### 2.4.2 Factors influencing population of bifidobacteria and the dominant fecal microflora

The factorial experimental design was used to determine the main factors influencing population of bifidobacteria and the dominant fecal microflora. Three factors including, infant ages (A), feeding types (B) and delivery mode (C). The levels of all factors were shown in Table 3. The results from the factorial experimental method analysis, mainly the *K* and *R* values, were calculated and were listed in Table 4. The population of bifidobacteria, clostridia, bacteroides and eubacteria was dependent on infant ages and feeding types. The impact of the factors on the population of bifidobacteria, clostridia, bacteroides and eubacteria in order: infant ages > feeding types > delivery modes, while the impact of the factors on the population of total bacteria in order: feeding types > delivery modes > infant ages. The infant ages and the feeding type were the main factors on the population of bifidobacteria, clostridia, bacteroides and eubacteria, while feeding type and delivery mode were the major factors on total bacteria population. The amount of bifidobacteria rapidly increased from first the week to 2 months of infant ages and remained stable after 2 months in all infants. Moreover, the amount of clostridia, bacteriodes and eubacteria significantly increased from first week to 5 months in all of infants ($P<0.05$) as shown in Table 5. Feeding type was also main factor influencing on population of bifidobacteria, clostridia, bacteroides, eubacteria and total bacteria. The exclusive breast feeding greatly supported the growth of bifidobacteria. The level of fecal bifidobacteria in both naturally born, exclusive breast fed infant (BF1-D) and Cesarean born, exclusive breast fed infant (BF2-E) was significantly higher than other infants ($P<0.05$). However, the highest level of bifidobacteria was found in naturally born, exclusive breast fed infant (BF1-D) from the first week to 5 months of ages (8.79 to 9.83 log CFU/g feces) as shown in Table 5 and

Figure 4. Interestingly, the amount of bifidobacteria in naturally born, formula fed infant (FF1-F) greatly increase (8.82 to 9.45 log CFU/g feces) after receiving the exclusive formula milk supplemented with *B. lactis* at 2 months to 5 months of age. In addition, using antibiotic (amoxicillin) in Cesarean born, combination fed infant (CF2-C) was significantly decreased the amount of bifidobacteria (9.21 to 8.77 log CFU/g feces) at 3 months of age. Formula milk was able to enhance the growth of clostridia and bacteroides. The highest level of the clostridia was found in Cesarean born, exclusively formula fed infant (FF1-F) (9.22 log CFU/g feces) when received exclusive formula milk supplemented with FOS and GOS at 2 months of age. However, the amount of clostridia was significantly decreased when received the exclusive formula milk supplemented with *B. lactis*. Conversely, low level of clostridia was found in both naturally born, combination fed infant (CF1-A) and naturally born, exclusively breast fed infants (BF1-D). In addition, the amount of bacteroides was significantly increased (8.59 to 9.19 log CFU/g feces) in Cesarean born, formula fed infant (FF2-H) at the first week to 5 months of age as shown in Table 5 and Figure 4. Moreover, the amount of total bacteria in naturally born, exclusively breast fed infant (BF1-D) (9.79 to 10.29 log CFU/g feces) and naturally born, exclusively formula fed infant (FF1-F) (9.94 to 10.13 log CFU/g feces) was significantly higher than another infants.

Table 4. Analysis of population of bifidobacteria, clostridia, bacteroides, eubacteria and total bacteria obtained from factorial experimental design.

| Bifidobacteria (log CFU/g feces) | | | | | |
|---|---|---|---|---|---|
| Level | Infant ages (A) | Level | Feeding types (B) | Level | Delivery modes (C) |
| $K_1$[a] | 8.70 | $K_1$[b] | 9.27 | $K_1$[c] | 9.15 |
| $K_2$ | 8.92 | $K_2$ | 8.98 | $K_2$ | 8.91 |
| K3 | 9.01 | $K_3$ | 8.84 | | |
| $K_4$ | 9.12 | | | | |
| $K_5$ | 9.16 | | | | |
| $K_6$ | 9.26 | | | | |
| R[b] | 0.57 | | 0.43 | | 0.24 |
| Optimal level | A6 | | B1 | | C1 |
| **Clostridia (log CFU/g feces)** | | | | | |
| Level | Infant ages (A) | Level | Feeding types (B) | Level | Delivery modes (C) |
| $K_1$ | 8.50 | $K_1$ | 8.40 | $K_1$ | 8.51 |
| $K_2$ | 8.50 | $K_2$ | 8.61 | $K_2$ | 8.60 |
| $K_3$ | 8.61 | $K_3$ | 8.61 | | |
| $K_4$ | 9.49 | | | | |
| $K_5$ | 8.56 | | | | |
| $K_6$ | 8.60 | | | | |
| R[b] | 0.99 | | 0.21 | | 0.09 |
| Optimal level | A4 | | B2, B3 | | C2 |
| **Bacteroides (log CFU/g feces)** | | | | | |
| Level | Infant ages (A) | Level | Feeding types (B) | Level | Delivery modes (C) |
| $K_1$ | 8.62 | $K_1$ | 8.82 | $K_1$ | 8.87 |
| $K_2$ | 8.95 | $K_2$ | 8.97 | $K_2$ | 8.92 |
| $K_3$ | 8.86 | $K_3$ | 8.90 | | |
| $K_4$ | 8.90 | | | | |
| $K_5$ | 9.01 | | | | |
| $K_6$ | 8.91 | | | | |
| R[b] | 0.39 | | 0.14 | | 0.05 |
| Optimal level | A5 | | B2 | | C2 |

[a] $k_i$ = (Σthe value of one factor at level $i$)/6, [b] $k_i$ = (Σthe value of one factor at level $i$)/3

[c] $k_i$ = (Σthe value of one factor at level $i$)/2

[b] $R$ = Max ($k_i$) – min ($k_i$) of one factor

Table 4. Analysis of population of bifidobacteria, clostridia, bacteroides, eubacteria and total bacteria obtained from factorial experimental design (continued).

| Eubacteria (log CFU/g feces) | | | | | |
|---|---|---|---|---|---|
| Level | Infant ages (A) | Level | Feeding types (B) | Level | Delivery modes (C) |
| $K_1^a$ | 9.26 | $K_1^b$ | 9.43 | $K_1^c$ | 9.43 |
| $K_2$ | 9.35 | $K_2$ | 9.43 | $K_2$ | 9.45 |
| $K_3$ | 9.43 | $K_3$ | 9.46 | | |
| $K_4$ | 9.50 | | | | |
| $K_5$ | 9.51 | | | | |
| $K_6$ | 9.58 | | | | |
| $R^b$ | 0.32 | | 0.03 | | 0.02 |
| Optimal level | A6 | | B3 | | C2 |

| Total bacteria (log CFU/g feces) | | | | | |
|---|---|---|---|---|---|
| Level | Infant ages (A) | Level | Feeding types (B) | Level | Delivery modes (C) |
| $K_1$ | 9.71 | $K_1$ | 9.88 | $K_1$ | 9.91 |
| $K_2$ | 9.84 | $K_2$ | 9.96 | $K_2$ | 9.72 |
| $K_3$ | 9.83 | $K_3$ | 9.61 | | |
| $K_4$ | 9.76 | | | | |
| $K_5$ | 9.87 | | | | |
| $K_6$ | 9.88 | | | | |
| $R^b$ | 0.17 | | 0.35 | | 0.19 |
| Optimal level | A6 | | B2 | | C1 |

[a]$k_i$ = (Σthe value of one factor at level $i$)/6, [b]$k_i$ = (Σthe value of one factor at level $i$)/3

[c]$k_i$ = (Σthe value of one factor at level $i$)/2

[b]$R$ = Max ($k_i$) – min ($k_i$) of one factor

Table 5. The amount of bifidobacteria and dominant fecal microflora in 6 healthy infants

| Delivery modes | Feeding types | Infant ages (months) | Bifidobacteria (log CFU/g feces) | Clostridium (log CFU/g feces) | Bacteroides (log CFU/g feces) | Eubacteria (log CFU/g feces) | Total bacteria (log CFU/g feces) |
|---|---|---|---|---|---|---|---|
| Natural born | Breast milk (BF1-D) | first week | $8.79 \pm 0.03^{f,I}$ | $8.22 \pm 0.02^{d,MN}$ | $8.47 \pm 0.06^{b,LM}$ | $9.29 \pm 0.10^{c,KL}$ | $9.79 \pm 0.04^{d,HI}$ |
| | | 1 | $9.13 \pm 0.08^{e,GH}$ | $8.25 \pm 0.08^{d,MN}$ | $8.85 \pm 0.08^{ab,J}$ | $9.32 \pm 0.07^{c,KL}$ | $10.13 \pm 0.07^{b,C}$ |
| | | 2 | $9.37 \pm 0.08^{c,CD}$ | $8.48 \pm 0.07^{ab,H}$ | $8.34 \pm 1.01^{b,M}$ | $9.67 \pm 0.06^{a,BC}$ | $10.22 \pm 0.06^{a,B}$ |
| | | 3 | $9.25 \pm 0.07^{d,DE}$ | $8.41 \pm 0.07^{bc,J}$ | $9.09 \pm 0.06^{b,G}$ | $9.66 \pm 0.05^{a,CD}$ | $9.79 \pm 0.09^{d,HI}$ |
| | | 4 | $9.52 \pm 0.03^{b,B}$ | $8.53 \pm 0.02^{a,G}$ | $9.33 \pm 0.05^{a,A}$ | $9.49 \pm 0.06^{b,H}$ | $10.03 \pm 0.06^{c,DE}$ |
| | | 5 | $9.83 \pm 0.02^{a,A}$ | $8.30 \pm 0.08^{cd,L}$ | $8.93 \pm 0.11^{ab,I}$ | $9.57 \pm 0.07^{ab,E}$ | $10.29 \pm 0.08^{a,A}$ |
| | Formula milk (FF1-F) | first week | $8.52 \pm 0.08^{e,J}$ | $8.67 \pm 0.06^{c,DE}$ | $8.42 \pm 0.06^{d,LM}$ | $9.35 \pm 0.06^{c,KL}$ | $10.13 \pm 0.03^{a,C}$ |
| | | 1 | $9.05 \pm 0.31^{c,H}$ | $8.98 \pm 0.19^{b,B}$ | $8.99 \pm 0.34^{b,H}$ | $9.55 \pm 0.13^{ab,EF}$ | $9.94 \pm 0.12^{c,FG}$ |
| | | 2 | $8.82 \pm 0.36^{d,I}$ | $9.22 \pm 0.21^{a,A}$ | $9.20 \pm 0.37^{a,BC}$ | $9.51 \pm 0.18^{b,FG}$ | $10.13 \pm 0.09^{a,C}$ |
| | | 3 | $9.35 \pm 0.03^{b,CD}$ | $8.53 \pm 0.04^{d,G}$ | $8.76 \pm 0.02^{c,K}$ | $9.66 \pm 0.08^{a,CD}$ | $10.00 \pm 0.02^{b,EF}$ |
| | | 4 | $9.34 \pm 0.13^{b,CD}$ | $8.66 \pm 0.14^{c,DE}$ | $8.96 \pm 0.12^{b,HI}$ | $9.38 \pm 0.08^{c,JK}$ | $10.08 \pm 0.10^{a,CD}$ |
| | | 5 | $9.45 \pm 0.11^{a,BC}$ | $8.69 \pm 0.16^{c,DE}$ | $9.27 \pm 0.16^{a,AB}$ | $9.51 \pm 0.05^{b,FG}$ | $10.13 \pm 0.12^{a,C}$ |
| | Combination milk (CF1-A) | first week | $9.16 \pm 0.01^{a,GH}$ | $8.42 \pm 0.03^{a,J}$ | $8.83 \pm 0.04^{abc,J}$ | $9.39 \pm 0.05^{a,J}$ | $9.72 \pm 0.11^{a,JK}$ |
| | | 1 | $9.13 \pm 0.04^{ab,GH}$ | $8.23 \pm 0.06^{b,MN}$ | $9.19 \pm 0.05^{a,BC}$ | $9.29 \pm 0.09^{abc,KL}$ | $9.72 \pm 0.08^{a,JK}$ |
| | | 2 | $8.80 \pm 0.06^{c,I}$ | $8.44 \pm 0.07^{a,HI}$ | $8.79 \pm 0.07^{abc,K}$ | $9.21 \pm 0.04^{bc,L}$ | $9.51 \pm 0.09^{d,NO}$ |
| | | 3 | $8.81 \pm 0.08^{c,I}$ | $8.27 \pm 0.06^{b,MN}$ | $8.56 \pm 0.05^{c,L}$ | $9.33 \pm 0.05^{a,KL}$ | $9.57 \pm 0.07^{c,MN}$ |
| | | 4 | $9.08 \pm 0.42^{b,H}$ | $8.30 \pm 0.08^{b,L}$ | $9.03 \pm 0.05^{ab,G}$ | $9.19 \pm 0.07^{c,L}$ | $9.67 \pm 0.08^{b,L}$ |
| | | 5 | $9.19 \pm 0.57^{a,FG}$ | $8.24 \pm 0.07^{b,MN}$ | $8.65 \pm 0.52^{bc,L}$ | $9.31 \pm 0.04^{ab,KL}$ | $9.58 \pm 0.12^{c,MN}$ |
| Caesarean born | Breast milk (BF2-E) | first week | $9.13 \pm 0.02^{b,GH}$ | $8.95 \pm 0.09^{a,B}$ | $8.65 \pm 0.09^{c,L}$ | $9.28 \pm 0.09^{b,KL}$ | $9.69 \pm 0.06^{a,L}$ |
| | | 1 | $9.40 \pm 0.07^{a,C}$ | $8.89 \pm 0.04^{a,BC}$ | $8.76 \pm 0.11^{bc,K}$ | $9.27 \pm 0.09^{b,KL}$ | $9.78 \pm 0.04^{a,I}$ |
| | | 2 | $9.27 \pm 0.31^{ab,D}$ | $8.81 \pm 0.05^{a,C}$ | $8.85 \pm 0.16^{b,J}$ | $9.27 \pm 0.08^{b,KL}$ | $9.67 \pm 0.07^{a,L}$ |
| | | 3 | $9.25 \pm 0.28^{ab,DE}$ | $8.77 \pm 0.09^{a,CD}$ | $9.12 \pm 0.12^{a,F}$ | $9.40 \pm 0.09^{b,J}$ | $9.74 \pm 0.08^{a,JK}$ |
| | | 4 | $9.17 \pm 0.27^{ab,GH}$ | $8.89 \pm 0.11^{a,BC}$ | $9.11 \pm 0.12^{a,F}$ | $9.63 \pm 0.11^{a,CD}$ | $9.77 \pm 0.07^{a,IJ}$ |
| | | 5 | $9.06 \pm 0.04^{b,H}$ | $8.87 \pm 0.14^{a,BC}$ | $8.35 \pm 0.11^{d,M}$ | $9.62 \pm 0.08^{a,CD}$ | $9.70 \pm 0.06^{a,L}$ |
| | Formula milk (FF2-H) | first week | $8.46 \pm 0.08^{c,J}$ | $8.14 \pm 0.05^{e,O}$ | $8.59 \pm 0.08^{c,L}$ | $8.71 \pm 0.31^{c,M}$ | $9.47 \pm 0.07^{b,O}$ |
| | | 1 | $8.81 \pm 0.15^{b,I}$ | $8.03 \pm 0.08^{f,P}$ | $8.71 \pm 0.07^{b,L}$ | $9.20 \pm 0.17^{b,L}$ | $9.93 \pm 0.24^{a,FG}$ |
| | | 2 | $8.90 \pm 0.14^{b,H}$ | $8.18 \pm 0.09^{d,N}$ | $9.14 \pm 0.06^{a,DE}$ | $9.23 \pm 0.14^{b,L}$ | $9.94 \pm 0.26^{a,F}$ |
| | | 3 | $9.12 \pm 0.06^{a,GH}$ | $8.61 \pm 0.04^{c,EF}$ | $9.21 \pm 0.16^{a,BC}$ | $9.67 \pm 0.06^{a,BC}$ | $9.86 \pm 0.11^{a,GH}$ |
| | | 4 | $9.09 \pm 0.08^{a, H}$ | $8.69 \pm 0.09^{b,DE}$ | $9.15 \pm 0.26^{a,D}$ | $9.66 \pm 0.09^{a,CD}$ | $9.94 \pm 0.12^{a,FG}$ |
| | | 5 | $8.84 \pm 0.06^{b,I}$ | $8.96 \pm 0.06^{a,B}$ | $9.19 \pm 0.23^{a,BC}$ | $9.71 \pm 0.08^{a,AB}$ | $9.92 \pm 0.14^{a,FG}$ |
| | Combination milk (CF2-C) | first week | $8.07 \pm 0.09^{d,K}$ | $8.60 \pm 0.60^{a,EF}$ | $8.74 \pm 0.10^{cd,K}$ | $9.56 \pm 0.12b^{c,EF}$ | $9.48 \pm 0.10^{d,O}$ |
| | | 1 | $8.01 \pm 0.04^{d,K}$ | $8.63 \pm 0.05^{a,EF}$ | $9.19 \pm 0.09^{ab,BC}$ | $9.44 \pm 0.17^{c,I}$ | $9.56 \pm 0.06^{c,MN}$ |
| | | 2 | $8.91 \pm 0.08^{b,I}$ | $8.52 \pm 0.04^{a,G}$ | $8.82 \pm 0.29^{c,J}$ | $9.65 \pm 0.09^{ab,CD}$ | $9.53 \pm 0.11^{cd,NO}$ |
| | | 3 | $9.21 \pm 0.09^{b,F}$ | $8.34 \pm 0.09^{b,K}$ | $8.67 \pm 0.08^{d,L}$ | $9.26 \pm 0.08^{d,KL}$ | $9.62 \pm 0.08^{b,LM}$ |
| | | 4 | $8.77 \pm 0.07^{c,I}$ | $8.32 \pm 1.97^{b,K}$ | $9.27 \pm 0.09^{a,AB}$ | $9.74 \pm 0.11^{a,B}$ | $9.73 \pm 0.12^{a,JK}$ |
| | | 5 | $9.19 \pm 0.05^{a,FG}$ | $8.55 \pm 0.07^{a,F}$ | $9.10 \pm 0.05^{b,G}$ | $9.75 \pm 0.09^{a,A}$ | $9.64 \pm 0.09^{b,LM}$ |

All data mean values of triple determination ± standard deviation (SD). Different superscript lowercase letters (a-d) indicate significant difference ($P < 0.05$) within the same column of each infant at first week to 5 months. Different superscript uppercase letters (A-O) indicate significant difference ($P < 0.05$) within the same column of all infants.

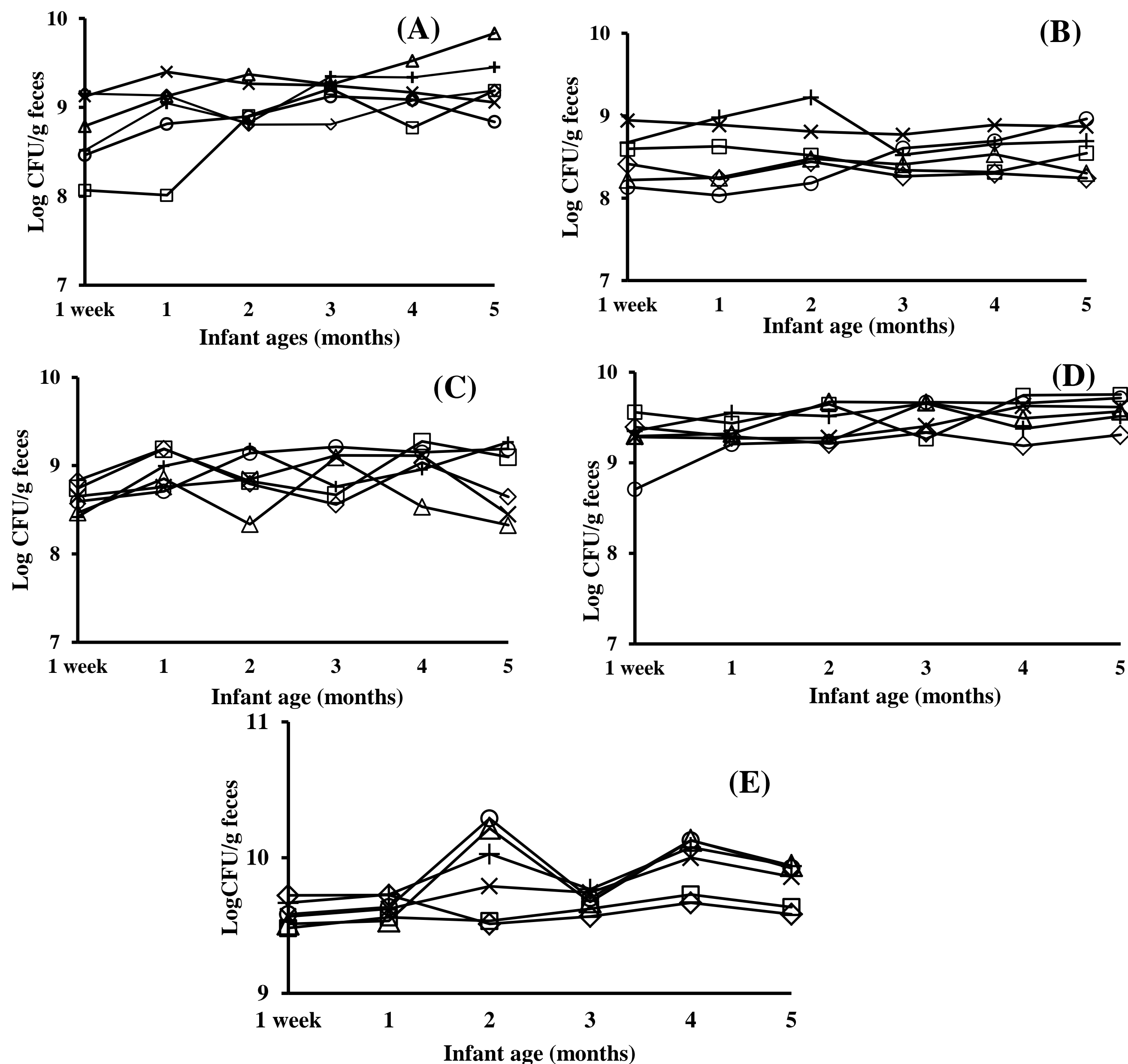


Figure 4. The amount of bifidobacteria (A), clostridia (B), bacteroides (C), eubacteria (D) and total bacteria (E) from feces of naturally born, combination fed infant (CF1-A) (◇), Cesarean born, combination fed infant (CF2-C) (□), naturally born, breast fed infant (BF1-D) (△), Cesarean born, breast fed infant (BF2-E) (×), naturally born, formula fed infant (FF1-F) (+) and Cesarean born, formula fed infant (FF2-H) (○).

**2.4.3 Determinants on the composition and diversity of fecal bifidobacteria**

The factorial experimental design was used to determine the main factors influencing composition of bifidobacteria. Three factors including, infant ages (A), feeding types (B) and delivery mode (C). The levels of the factors studied were shown in Table 3 were evaluated using factorial experimental design. The PCR-DGGE technique target V2-V4 region of bacterial 16S rDNA was applied to reveal bifidobacteria in the infant feces (Figure 5 and Table 6). Seventeen to thirty-six DGGE band of bifidobacteria were detected in 6 healthy infants. The results from the factorial experimental method analysis, mainly the *K* and *R* values, were calculated and were listed in Table 7. The impact of the factors on the composition of bifidobacteria in order: feeding type > delivery mode > infant age. DGGE analysis demonstrated the changes in the bifidobacterial composition associated with feeding type and delivery mode. The highest number of bifidobacterial bands was observed in both naturally born, exclusively breast fed infant (BF1-D) and naturally born, combination of breast and formula fed infants (CF1-A) as shown in Table 8. However, the highest number of bifidobacterial species was observed in both naturally exclusively formula fed infant (FF1-F) and naturally born, combination of breast and formula fed infants (CF1-A) (Figure 5, Table 9). Furthermore, many species of bifidobacteria including *B. adolescentis* (band 5), *Bifidobacterium sp*. (band 4, 6,18, 19, 21 and 24), *B. longum* (band 11 ) *B. catenulatum* (band 12 ) and *B. breve* (band 14) were able to persist from the first week to 5 months in naturally born, both of breast fed (BF1-D) and combination fed infants (CF1-A). Interestingly, *B. lactis* (band 38) was detected at 3 and 4 months of naturally exclusively formula fed infant (FF1-F). This evidence supported that *B. lactis* from formula milk was able to colonize in infant. However, this strain was not persistent in FF1-F infant after received this formula milk supplemented with *B. lactis* at 5 months of age. There was only *B. longum* (band 35) was able to persist from first week to 5 months of FF1-F infant. Antibiotic (amoxicillin) treatment for 7 days in Cesarean born, combination fed infant (CF2-C) did not affect bifidobacterial composition, *Bifidobacterium sp.* (band 2, 9) *B. adolescentis* (band 5) *B. bifidum* (band 7, 8) were able to detect before and after amoxicillin treatment.

The most common bifidobacterial species in healthy infants were *Bifidobacterium* sp. and *B. longum* (Table 9). In addition, *B. breve* (band 14), *Bifidobacterium* sp. (band 18, 19, 21, 24), *B. longum* (band 22) were predominant in naturally born, exclusively breast fed infant (BF1-D). Moreover, uncultured *Bifidobacterium* sp. (band 4, 6), *B. adolescentis* (band 5) *B. longum* (band 11) were frequently detected in naturally born, combination of breast and formula fed infant (CF1-A). These strains were abundantly detected and remained persistently in both naturally born, exclusively breast fed infant and naturally born, combination of breast and formula fed infant. Interestingly, multiple bifidobacterial strains including uncultured *Bifidobacterium* sp. (band 2, 4, 6, 18, 19, 20, 21, 24, 25), *B. adolescentis* (band 5) *B. bifidum* (band 7, 8), *B. dentium* (band 10), *B. longum* (band 11), *B. breve* (band 14), *B. longum* (band 22) were detected at the first week and able to persist until 5 months of ages. The majority of these strains were able to detect in infant who received breast milk. This result suggested that breast milk feeding was able to support colonization and persistence of bifidobacteria.

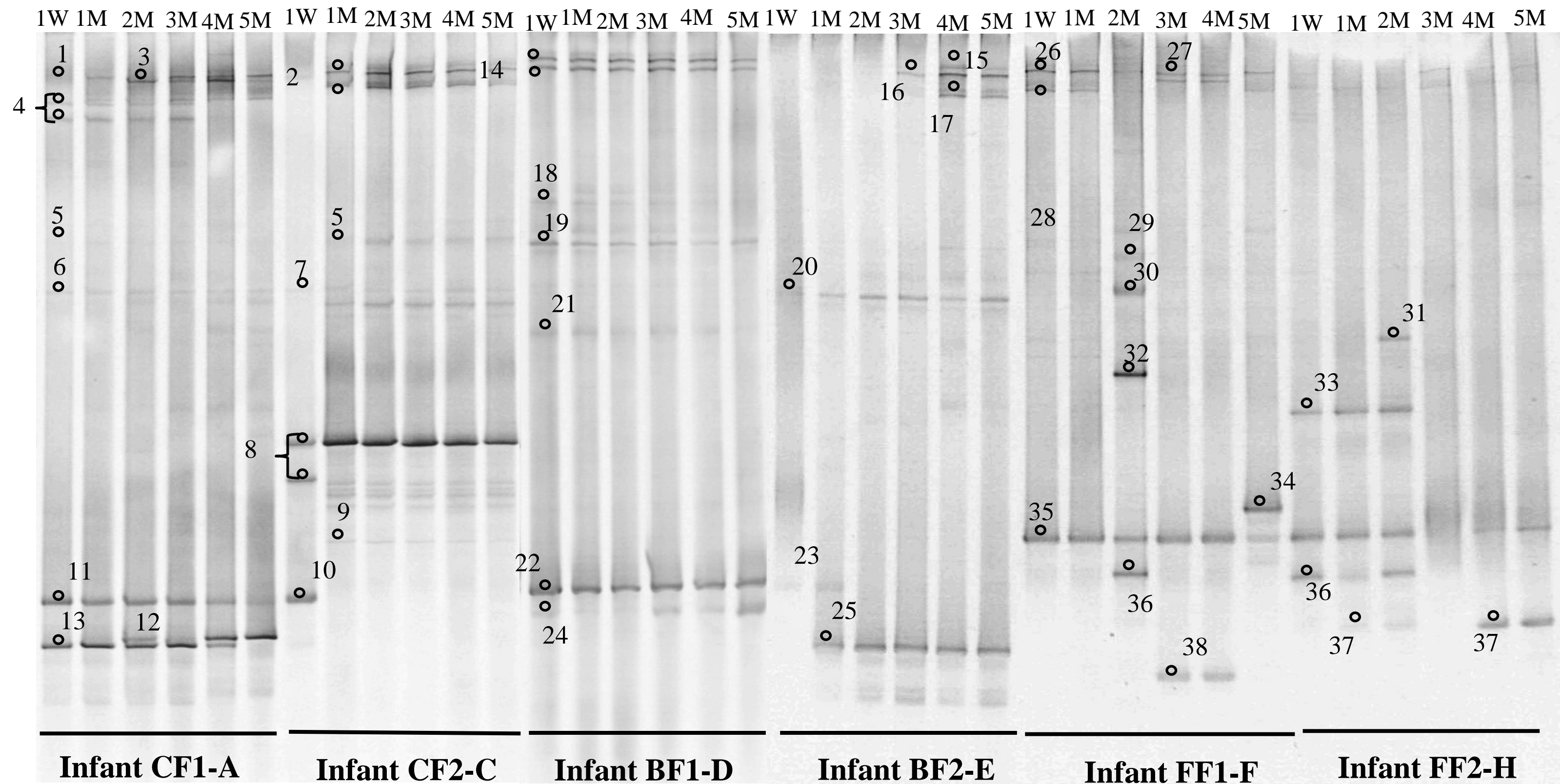


Figure 5. PCR-DGGE profile of bifidobacteria from naturally born, combination fed infant (CF1-A), Cesarean born, combination fed infant (CF2-C), naturally born, breast fed infant (BF1-D), Cesarean born, breast fed infant (BF2-E), naturally born, formula fed infant (FF1-F) and Cesarean born, formula fed infant (FF2-H) at the first week to 5 months of age.

Table 6. Strains identification of bifidobacterial diversity in 6 healthy infants

| Factors | Natural born | | | Cesarean born | | |
|---|---|---|---|---|---|---|
| | **Combination milk (CF1-A)** | **Breast milk (BF1-D)** | **Formula milk (FF1-F)** | **Combination milk (CF2-C)** | **Breast milk (BF2-E)** | **Formula milk (FF2-H)** |
| **1 week** | *B. callitrichos* (1)<br>Uncultured *Bifidobacterium* sp. (4) (6)<br>*B. adolescentis* (5)<br>*B. longum* (11)<br>*B. pseudocatenulatum* (13) | *B. breve* (14)<br>*Bifidobacterium* sp. (18),(19),(21),(24)<br>*B. longum* (22) | Uncultured *Bifidobacterium* sp. (26)<br>*Bifidobacterium* sp. (28)<br>*B. longum* (35) | *B. bifidum* (7),(8)<br>*B. dentium* (10) | Uncultured *Bifidobacterium* sp. *(20)*<br>*B. dentium* (23) | Uncultured *Bifidobacterium* sp. (33)<br>*B. longum* (35)<br>*B. pseudocatenulatum (36)* |
| **1 month** | *B. callitrichos* (1)<br>Uncultured *Bifidobacterium* sp. (4) (6)<br>*B. adolescentis* (5)<br>*B. longum* (11)<br>*B. pseudocatenulatum* (13) | *B. breve* (14)<br>*Bifidobacterium* sp. (19),(21),(24)<br>*B. longum* (22) | Uncultured *Bifidobacterium* sp. (26)<br>*Bifidobacterium* sp. (28)<br>*B. longum* (35) | *Bifidobacterium* sp. (2), (9)<br>*B. adolescentis* (5)<br>*B. bifidum* (7),(8) | Uncultured *Bifidobacterium* sp. (20)<br>*Bifidobacterium* sp. (25) | Uncultured *Bifidobacterium* sp. (33)<br>*B. longum* (35)<br>*B. pseudocatenulatum* (36)<br>*B. kashiwanohense* (37) |
| **2 months** | Uncultured *Bifidobacterium* sp. (3),(4), (6)<br>*B. adolescentis* (5)<br>*B. longum* (11)<br>*B. catenulatum* (12) | *B. breve* (14)<br>*Bifidobacterium* sp. (19),(21),(24)<br>*B. longum* (22) | Uncultured *Bifidobacterium* sp. (26), (30)<br>Uncultured bacterium (29)<br>*B. adolescentis* (32)<br>*B. longum* (35) | *Bifidobacterium* sp. (2), (9)<br>*B. adolescentis* (5)<br>*B. bifidum* (7),(8) | Uncultured *Bifidobacterium* sp. (20)<br>*Bifidobacterium* sp. (25) | *Bifidobacterium* sp. (31)<br>Uncultured *Bifidobacterium* sp. (33)<br>*B. longum* (35)<br>*B. pseudocatenulatum* (36)<br>*B. kashiwanohense* (37) |

Table 6. Strains identification of bifidobacterial diversity in 6 healthy infants (continued)

| Factors | Natural born | | | Cesarean born | | |
|---|---|---|---|---|---|---|
| | **Combination fed (CF1-A)** | **Breast fed (BF1-D)** | **Formula fed (FF1-F)** | **Combination fed (CF2-C)** | **Breast fed (BF2-E)** | **Formula fed (FF2-H)** |
| **3 months** | Uncultured *Bifidobacterium* sp. (3),(4), (6) | *B. breve* (14) | *B. pseudocatenulatum* (36) | *Bifidobacterium* sp. (2), (9) | Uncultured *Bifidobacterium* sp. (20), *(16)* | *B. longum* (35) |
| | *B. adolescentis* (5) | *Bifidobacterium* sp. (19),(21),(24) | Uncultured *Bifidobacterium* sp. (27) | *B. adolescentis* (5) | *Bifidobacterium* sp. (25) | |
| | *B. longum* (11) | *B. longum* (22) | *B. longum* (35) | *B. bifidum* (7),(8) | | |
| | *B. catenulatum* (12) | | *B. animalis* subsp. *lactis* (38) | | | |
| **4 months** | Uncultured *Bifidobacterium* sp. (3) (6) | *B. breve* (14) | Uncultured *Bifidobacterium* sp. (27) | *Bifidobacterium* sp. (2), (9) | Uncultured bacterium (15) | *B. longum* (35) |
| | *B. adolescentis* (5) | *Bifidobacterium* sp. (19),(21),(24) | *B. longum* (35) | *B. adolescentis* (5) | *Bifidobacterium* sp. (17), (25) | *B. kashiwanohense* (37) |
| | *B. longum* (11) | *B. longum* (22) | *B. animalis* subsp. *lactis* (38) | *B. bifidum* (7),(8) | Uncultured *Bifidobacterium* sp. (20) | |
| | *B. catenulatum* (12) | | | | | |
| **5 months** | Uncultured *Bifidobacterium* sp (3), (6) | *B. breve* (14) | Uncultured *Bifidobacterium* sp. (27) | *Bifidobacterium* sp. (2), (9) | Uncultured bacterium (15) | *B. longum* (35) |
| | *B. adolescentis* (5) | *Bifidobacterium* sp. (19),(21),(24) | *B. dentium* (34) | *B. adolescentis* (5) | Uncultured *Bifidobacterium* sp. (20) | *B. kashiwanohense* (37) |
| | *B. longum* (11) | *B. longum* (22) | *B. longum* (35) | *B. bifidum* (7),(8) | *Bifidobacterium* sp. (17), (25) | |
| | *B. catenulatum* (12) | | | | | |

Table 7. Analysis of the number of band of bifidobacteria obtained from factorial experimental design.

| Level | Infant ages (A) | Level | Feeding type (B) | Level | Delivery mode (C) |
|---|---|---|---|---|---|
| $K_1^a$ | 4.50 | $K_1^b$ | 4.83 | $K_1^c$ | 5.00 |
| $K_2$ | 4.33 | $K_2$ | 3.08 | $K_2$ | 3.72 |
| $K_3$ | 5.00 | $K_3$ | 5.17 | | |
| $K_4$ | 4.00 | | | | |
| $K_5$ | 4.17 | | | | |
| $K_6$ | 4.17 | | | | |
| $R^b$ | 0.83 | | 2.08 | | 1.28 |
| Optimal level | A3 | | B3 | | C1 |

[a]$k_i$ = (Σthe value of one factor at level $i$)/6, [b]$k_i$ = (Σthe value of one factor at level $i$)/3

[c]$k_i$ = (Σthe value of one factor at level $i$)/2

[b]$R$ = Max ($k_i$) – min ($k_i$) of one factor

Table 8. The number of DGGE band of bifidobacteria from 6 healthy infants.

| Infant ages (months) | Natural born | | | Cesarean born | | |
|---|---|---|---|---|---|---|
| | Breast milk BF1-D | Formula FF1-F | Combination CF1-A | Breast milk BF2-E | Formula FF2-H | Combination CF2-C |
| 1 week | 6 | 3 | 6 | 1 | 3 | 3 |
| 1 | 6 | 3 | 6 | 2 | 4 | 5 |
| 2 | 6 | 5 | 6 | 3 | 5 | 5 |
| 3 | 6 | 3 | 6 | 3 | 1 | 5 |
| 4 | 6 | 3 | 5 | 4 | 2 | 5 |
| 5 | 6 | 3 | 5 | 4 | 2 | 5 |
| Total | 36 | 20 | 34 | 17 | 17 | 28 |

Table 9. The composition of bifidobacteria in 6 healthy infants determined using PCR-DGGE.

| Bifidobacterial species | CF1-A | CF2-C | BF1-D | BF2-E | FF1-F | FF2-H |
|---|---|---|---|---|---|---|
| *B. callitrichos* | * | | | | | |
| *Bifidobacterium* sp. | * | * | * | * | * | * |
| Uncultured *Bifidobacterium* sp. | * | | | * | * | * |
| *B. adolescentis* | * | * | | | * | |
| *B. bifidum* | | * | | | | |
| *B. dentium* | | * | | * | | * |
| *B. longum* | * | | * | | * | * |
| *B. catenulatum* | * | | | | | |
| *B. pseudocatenulatum* | * | | | | * | * |
| *B. breve* | | | * | | | |
| *B. dentium* | | | | * | * | |
| *B. kashiwanohense* | | | | | | * |
| *B. animalis* subsp. *lactis* | | | | | * | |
| Total of bifidobacterial species | 7 | 4 | 3 | 4 | 7 | 6 |

***** Bifidobacterial species can detect in each infant

**2.5 Discussion**

The infant gut is thought to be sterile at birth. Initial colonization and early establishment of the infant gut is influenced by delivery mode, antibiotic treatment, geographical zone, infant age, and feeding type (Penders *et al*., 2006; Thompson-Chagoyan *et al*., 2007; Marques *et al*., 2010). This study demonstrated that infant age and feeding type were found to be the most influential factors relating to the number of total bifidobacteria, clostridia, bacteriodes and eubacteria. The significantly bacterial level change with age, the age-affiliated microbiota population shifts from 3 days to 2 years after birth (Rigon *et al*., 2012; Odamaki *et al*., 2016). The amount of bifidobacteria rapidly increased from the first week to 2 months of infant age and stable then after 2

months in all of infants. Moreover, the amount of clostridia, bacteriodes and eubacteria significantly increased from the first week to 5 months in all of infants. Bifidobacteria is abundant in the stool of most infants during the initial stay of life, especially at an age 1 week-3 months (Harmsen *et al.*, 2000; Rutayisire *et al.*, 2016). In addition, the development of the infant intestinal microflora has been shown to be heavily dependent on feeding type in the first week/months of life (Orrhage *et al.*, 1999; Fanaro *et al.*, 2003). Feeding type affected the concentration of different microbe (Penders *et al.*, 2006). This study revealed that exclusively breast feeding promoted the growth of bifidobacteria. Likewise, many previous studies showed the dominant bifibacteria in the exclusively breast fed infant (Harmsen *et al.*, 2000; Tannock *et al.*, 2013). Moreover, our result demonstrated that the abundance of bifidobacteria in exclusively breast fed infant was higher than both of formula fed infant and combination of breast and formula fed infant, while the levels of clostridia and bacteriodes were lower. Breast milk guided the development of the infant gut microbiota by preventing pathogen colonization and it also contained high level of human milk oligosaccharide (HMO) that selectively stimulate growth of bifidobacteria (Coppa *et al.*, 2006). HMO is able to resistant to human intestinal digestive enzymes and therefore acts as substrate for fermentation in the distal gut and leading to low pH in the gut, such condition growth of bifidobacteria, but is inhibitory to many other bacteria, such as clostridia and bacteriodes (Tham *et al.*, 2011). Moreover, a number of peptides capable of stimulating the growth of several bifidobacteria have recently been isolated from human milk. (Coppa and Gabrielli, 2008). The authors reported that infants would ingest between $1\times10^{5}$-$1\times10^{7}$ bacteria a day from consuming approximately 800 mL/day of breast milk. Moreover, breast milk from healthy women was reported to contain bifidobacteria approximately $3.35\times10^{3}$ bacterial cell/ml in healthy mothers (Martin *et al.*, 2009). *B. longum* was the predominant species, followed by *B. catenulatum*, *B. bifidum* and *B. breve* (Gueimonde *et al.*, 2007). Bifidobacteria residing in maternal gut could possibly colonize mammary gland through an endogenous route (the so-called entero-mammary pathway), involving maternal dendritic cells and macrophages (Martin *et al.*, 2004; Rodriguez, 2014). According to this

study, feeding type and delivery mode were the most influential factors on bifidobacterial composition and diversity. Likewise, several studies demonstrated that the most important determinants of the gut microflora composition in infants were feeding type and delivery mode. (Pender *et al*., 2006; Wall *et al*., 2009). However, age of infant was also important determinants to shape gut microflora composition in infants. Our study demonstrated that several strains of bifidobacteria could colonize in infant begining at the first week of age and remain quite stable after 1 month of age. The first days and weeks of age represent a crucial window of opportunity for shaping the development of GI tract (Houghteling and Walker, 2015). Moreover, the gut microbiota is less stable than later in childhood and there are changes in the phylogenetic diversity in the first month of age (Nuriel-Ohayon *et al*., 2016). In addition, the number of DGGE bands in natural born infants was higher than cesarean born infants. The highest of the number of bifidobacteria band was observed in both naturally born, exclusively breast fed infant (BF1-D) and naturally born, combination of breast and formula fed infants (CF1-A). The infant could expose to the bacteria from mother's vagina and maternal intestine during vaginal delivery. (Mikami *et al*., 2009). Several studies demonstrated vertical transfer of bifidobacteria from the mother's birth canal to the newborn during delivery (Tannock *et al*., 1990; Gronlund *et al*., 2007). The feeding type of infant affect the composition of microbiota within the gut of the infant and several studies indicated that there is difference in the composition of microbes in the gut of infants on different feeding type (Fanaro *et al*., 2003; Pander *et al*., 2006).

According to this study, the highest of the number of bands was observed in both naturally born, exclusively breast fed infant (BF1-D) and naturally born, combination of breast and formula fed infants (CF1-A). This evidence corresponded with persistence of several bifidobacterial species included *B. adolescentis, Bifidobacterium sp*. *B. longum, B. catenulatum* and *B. breve* from these infants. In agreement with previous studied demonstrated that bifidobacteria most frequently isolated from naturally born, exclusively breast fed infants belong to the spices *B. bifidum*, *B. longum* and *B. breve* (Mitsou *et al*., 2008; Solis *et al*., 2010; Turroni *et al*., 2012). The bifidobacterial predominance in the

intestinal microbiota of breast fed infants has been linked to the direct transfer of maternal bifidobacteria to newborns in breast milk (Gronlund *et al*., 2007). *B. longum*, *B. bifidum* and *B. breve* were the most widely detected in breast milk (Gueimonde *et al*., 2007; Gronlund *et al*., 2007; Jost *et al*., 2012; Soto *et al*., 2014). Moreover, natural prebiotic in breast milk selectively support growth and diversity of bifidobacteria (Satokari *et al*., 2002; Chen *et al*., 2007; Sela and Mills, 2010; Fernandez *et al.*, 2013). In addition, *B. breve, B. bifidum, and B. longum* has shown that these species are efficient with regard to the use of intact HMO (Locascio *et al*., 2009; Ruiz-Moyano *et al*., 2013). Furthermore, the number of band and the number of bifidobacterial species was also high in CF1-A infant. This may be because the addition of oligosaccharide in formula milk induced breast-fed infants-similar microbiota colonization in formula-fed infants as supported by (Rinne *et al*., 2005; Fernandez *et al*., 2013). Moreover, the microbiota of naturally born, combination of breast and formula fed infant (CF1-A) contained relatively more *B. longum*, *B. catenulatum* and *B. adolescentis*, which *B. catenulatum* and *B. adolescentis* species that are commonly found in adults (Matsuki *et al*., 1999; Voreades *et al*., 2014; Arboleya *et al*., 2016). In addition, naturally born, formula fed infant (FF1-F) and naturally born, combination of breast and formula fed infants (CF1-A) exhibited more divers bifidobacteria species than another infants. This result was consistent with previous studies (Lee *et al*., 2015; Thompson *et al*., 2015).

Formula milk supplemented with *B. lactis* was able to modulate the population and the composition of the bifidobacteria of FF1-F infant. In our study, *B. lactis* was detected at 3 and 4 months of FF1-F infant that related with changing of formula milk, the Hi-Q one plus brand to Nan HA 1 brand that supplemented with *B. lactis* of FF1-F infant. Supplementation of formula milk with probiotic strain is one of the strategies commonly considered to improve the gut microflora in GI tract. Several studies have demonstrated that *B. lactis* was able to improve gastrointestinal health (Saavedra *et al*., 2004; Braegger *et al*., 2011; Baglatzi *et al*., 2016). However, this strain could not persist after 4 month. Only *B. longum* was able to persist from first week to 5 months. Moreover, using amoxicillin in Cesarean born, combination fed infant (CF2-C) was decreased the

amount of bifidobacteria, while did not affect bifidobacterial composition and species of bifiobacteria including *Bifidobacterium* sp., *B. adolescentis* and *B. bifidum.* Interestingly, multiple bifidobacterial strains including uncultured *Bifidobacterium* sp., *B. adolescentis*, *B. bifidum*, *B. dentium*, *B. longum*, *B. breve*, *B. longum* were detected at first week and able to persist in both of naturally born, exclusively breast fed infants (BF1-D) and cesarean born, exclusively breast fed infants (BF2-E) and naturally born, combination of breast and formula fed infants (CF-A) and cesarean born, combination of breast and formula fed infants. These strains could persist throughout and exhibit strong mucosal adhesive properties as supported by Barrett *et al*. (2015).

**2. 6 Conclusion**

Feeding type and infant age were the most influential factors on the abundances of bifidobacteria, clostridia, bacteriodes and eubacteria. The highest of the amount of bifidobacteria was found in naturally born, exclusive breast fed infant. Furthermore, feeding type and delivery mode were the most influential factors on bifidobacterial composition and diversity. The number of DGGE bands in naturally born, exclusive breast fed infant and naturally born, combination fed infant. Moreover, the number of bifidobacterial species was observed in naturally born, formula fed infant and naturally born, combination fed infant. Several species of bifidobacteria included *B. adolescentis, Bifidobacterium sp*. *B. longum, B. catenulatum* and *B. breve* were able to persistent from first week to 5 months in naturally born, both of breast fed infant and combination fed infants. The most common bifidobacterial species in healthy infants were *Bifidobacterium* sp. and *B. longum.*

# CHAPTER 3

# EFFECT OF FEEDING TYPES ON POPULATION AND DIVERSITY OF LACTIC ACID BACTERIA IN THAI HEALTHY NEWBORNS

## 3.1 Abstract

The population level and diversity of lactic acid bacteria (LAB) present in the feces of infants were compared between breast-, formula- and combined-feeding types. There were two infant formulae with FOS-GOS and inulin-GOS supplementations. The number of fecal LAB from the infants fed with the combination diet of breast milk and a formula containing FOS-GOS (CF1-A infant) and inulin-GOS (CF2-C infant) was significantly higher than that from both exclusively breast-fed (BF1-D infant and BF2-E infant) and exclusively formula fed ones (FF1-F infant and FF2-H infant) ($P<0.05$). According to PCR-DGGE analysis, the band richness of LAB population in infants with combination (CF1-A infant and CF2-C infant) and exclusive formula feeding (FF1-F infant and FF2-H infant) and the exclusively breast-fed infants (BF2-E) was not different, while the number of LAB in exclusively breast fed infant (BF1-D) was lower than another. Type of feeding had not a significant influence on LAB diversity. Fourteen species of LAB were found from 6 infants and each infant presented 4-6 species of LAB and the most number of LAB species was found in the infant with the exclusive breast fed infant (BF2-E infant) and exclusive formula fed diet supplemented with FOS-GOS (FF1-F infant). Moreover, uncultured bacteria were commonly found in the commensal intestinal microbiota in Thai infants, independently on feeding type. They were detected abundantly in feces of all infants. Combination feeding was able to support the growth of *L. plantarum* and *Leu. mesenteroides.* The exclusive feeding with formula supplemented with FOS-GOS enhance growth of *L. rhamnosus* and *L. gasseri*, while the formula supplemented with inulin-GOS sustained growth of *L. gasseri*, *L. fermentum* and *L. paracasei.* In addition, exclusive breast milk feeding was capable to promote the growth of *L. gasseri*, *L. crispatus*, *L. plantarum* and *L. helveticus*.

## 3.2 Introduction

The neonatal period is crucial for intestinal colonization, which influences the adult intestinal microbiota and lifelong (Morelli, 2008). The infant gut is thought to be sterile at birth. (Zetterstrom *et al*., 1994; Mackie *et al*., 1999; Tannock *et al*., 2000). The critical stage of gut colonization occurs within the first few weeks after birth and is influenced by a variety of factors including gestational age, delivery mode, hygiene, antibiotic use, geographical zone and feeding type (Thompson-Chagoyan *et al*., 2007; Marques *et al*., 2010). In early life, one of the primary drivers shaping gut microbiota structures and changes that occur during infancy is type of feeding (Penders *et al*., 2006; Thum *et al*., 2012; Voreades *et al*., 2014). This factor has a significant influence on the relative proportions of bacteria that establish in the infant gut by providing substrates for bacterial proliferation and function (Edward and Parrett, 2002; Guaraldi and Salvatori, 2012). Several studies have reported that bifidobacteria and lactic acid bacteria (LAB) dominate microbiota of breast-fed infants, while formula-feeding generally results in a more diverse microbial population, including *Bifidobacterium, Bacteroides*, *Clostridium* and *Streptococcus* (Moro *et al*., 2002; Penders *et al*., 2006).

LAB constitutes a large group of non-sporulating, Gram-postive, catalase and oxidize-negative rods and cocci. They play an important role in various physiological functions and widely distributed in intestinal tracts of human body (Gibson and Roberfroid, 1995). LAB was able to inhibit growth of pathogens by lowering the pH, due to the production of lactic and acetic acid or by competing for nutrients and epithelial adhesion site and to be beneficial for maintaining host health (Holzapfel *et al*., 1998). Furthermore, LAB can infiltrate an infant's sterile digestive tract by means of contact with the mucosal surface of the mother's vagina or from the mother's breast milk and environment (Harmsen *et al*., 2000; Mitsou *et al*., 2008; Solis *et al*., 2010; Martin *et al*., 2012). *Lactobacillus fermentum, Lactobacillus casei, Lactobacillus paracasei, Lactobacillus delbrueckii, Lactobacillus gasseri and Lactobacillus plantarum* were frequently isolated from infants (Ahrne *et al*., 2005; Haarman and Knol, 2006).

The influence of feeding type, breast-fed, formula-fed and combination-fed, on the composition of the LAB is presently equivocal. Several reports maintain that there would be no significant difference when the formula milk is supplemented with prebiotic which can promote the colonization of LAB, thus making the gut LAB similar to that of breast-fed infant (Magne1 *el al*., 2006; Boehm and Moro, 2008). In contrast, other studies reported that all of the infants were colonized by LAB, with differences in the prevalence or abundance between breast-fed and formula-fed infants (Harmsen *et al*., 2000; Hascoet *et al*., 2011; Wang *et al*., 2015). Therefore, the goal of this study was to compare LAB population and diversity between breast-fed, formula-fed and combination-fed infants.

## 3.3 Materials and Methods

### 3.3.1 Subjects and infant fecal sample preparation

The protocol of this study was reviewed and approved by the ethics committee of faculty of medicine, Prince of Songkla University (EC Number: 55-244-19-2-3). Six healthy, full-term infants were included in this study (Table 10). All infants were born at 37 to 42 weeks of gestation with no evidence of disease at birth and no antibiotic treatment. Two healthy infants were fed with a combination diet of breast milk and a formula either containing FOS-GOS (CF1-A infant) or inulin-GOS (CF2-C infant). Another two infants were exclusively fed on breast milk (BF1-D infant and BF1-E infant). The others were exclusively fed on a commercial formula supplemented with either FOS-GOS (FF1-F infant) or inulin-GOS (FF2-H infant). All infants showed no sign of any health problem from birth until to the end of this study. Fecal samples were collected every month from infants beginning from the first week after birth up to 5 months. The sample collected in a sterile plastic container was then immediately cooled and strictly maintained at 4 $^{o}$C throughout transportation period (not longer than 1 h). All fecal samples were kept frozen at -20 $^{o}$C.

### 3.3.2 LAB population presents in infant feces using fluorescent *in situ* hybridization (FISH) technique

Homogenized infant fecal sample (375µl) were added to 1.125 ml of 4% (w/v) filter-sterilized paraformaldehyde solution (Acros, New Jersey, USA) and left overnight at 4°C. The mixture was thereafter centrifuged at 10,000 rpm for 5 min at 4 ºC. The fixed cells were washed twice with cold filter sterilized phosphate-buffered saline (PBS 0.1 M, pH 7.2), and then resuspended in 300 µl of PBS/99% ethanol (1:1(w/v) mixture and stored at -20 ºC until further analysis, which was no longer than 3 months Uraipan *et al*., 2014). FISH technique was performed as described by Hongpattatakere *et al.* (2012). The genus-specific 16S rRNA target oligonucleotide probe (Sigma, USA) labeled with the fluorescent dye Cy3 used was Lab158 (5'-GGTATTAGCA(T/C)CTGTTTCCA-3') and nucleotide target 4',6-diamidino-2-phenylindole (DAPI) dye (Sigma, St. Louis, MO, USA) was used for total bacterial counts. Cell were counted using fluorescence microscope Nikon Eclipse 80i, USA) with a minimum of 15 fields, 10-100 cells/field). The number of LAB present in each sample was determined using the following equation:

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

Where 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. The dilution of the sample used with a particular probe (e.g. 10× for Lab158 count); the area of the well divided by the area of the microscopic field; and the conversion factor taking the number based on milliliter of sample (Ogue-Bon *et al*., 2010).

### 3.3.3 LAB diversity presents in infant feces using denaturing gradient gel electrophoresis (DGGE) technique.

#### 3.3.3.1 Extraction of genomic DNA from fecal samples

Infant fecal sample 1 g was washed in 1 ml with PBS then was centrifuged each preparation at 10,000 rpm for 5 min in order to reduce the PCR inhibitors. The pellet of

fecal sample were resuspended in 100 µl of distilled water, serially diluted in 100 µl of 1% Triton X-100 (Fluka, Seelze, Germany), heated at 100 °C for 5 min, and immediately cooled in ice water (Wang *et al*., 1996). The samples were resuspended in 100 µl of lysis buffer (6.7% sucrose, 50 mM TrisHCl (pH 8.0), 10 mM EDTA, 20 mg of lysozyme/ml (Invitrogen, CA, USA), 1,000 U of mutanolysin/ml (Sigma-Aldrich, Steinheim, Germany), 100 mg of RNaseA/ml) (Amresco, Ohio, USA) according to Walter *et al.* (2001).

### 3.3.3.2 Nested PCR-DGGE analysis

In order to increase sensitivity and to facilitate the DGGE analyses, a nested PCR was performed to amplify the target DNA prior to DGGE analysis. The first round PCR was performed using the universal forward and reverse primers of 27-f (5'-AGAGTTTGATCMTGGCTCAG-3'and 1492-r (5'-TACGGYTACCTTGTTACGACTT-3'), respectively (Dees *et al*., 2001). The PCR volumes of 50 µl contained 25 µl red dye PCR master mix (GeNei, Bangalore, India), 5 µL of each primer (2µM), 1 µL of genomic DNA and 14 µL of sterile Milli-Q water (Omnipur, Gibbstown, NJ, USA). The first round PCR products were used as templates in the next round of the amplification. The variable V3 region (380 bp) of 16S rDNA of LAB was targeted using Lac1-f (5'-AGCAGTAGGGAATCTTCCA-3') and Lac2-GC-r (5'CGCCCGGGGCGCGCCCCGG GCGGCCCGGGGGCACCGGGGGATTYCACCGCTACACATG-3') (Water *et al*., 2001). DGGE analysis of the PCR amplicons as carried out on the DGGE system (Cleaver Scientific, York, UK) under the condition described previously (Uraipan *et al*., 2014). The linear denaturing gradients of urea and formamide use for separation of amplicons from LAB were 30-55%. A 100% denaturing solution contained 40% (v/v) formamide (Amresco, Ohio, USA) and 7 M urea (Acros, New Jersey, USA). The electrophoresis was conducted with a constant voltage of 70 V at 60$^{o}$C for about 16 h (Vanhoutte *et al*., 2004). After electrophoresis, the gels were incubated with 1× SYBR® Gold (Invitrogen, Grand Island, NY, USA) for 30 min, before being viewed under UV light transillumination (Alpha innotech corporation, San Leandro, USA). The LAB

diversity was evaluated by the number of bands. Moreover, specie level was determined using the following equation: A/B $\times$ 100 where A; the number of the bands of each species in each individual and B; total of visible bands present in each infant.

The DGGE bands were cut under Ultra Bright LED Transilluminator (Gellex, Hsinchu City, Taiwan). Each individual DNA band was extracted by incubating in 100 µl of 1×TE buffer overnight at 4 ºC. The obtained DNA was subsequently re-amplified by PCR using Lac1-f and Lac2-r primers without GC clamp, before its nucleotide sequence was determined by Ward Medic Ltd., Part. (1$^{st}$ Base Distributor, Thailand). Closest matches for partial 16S rRNA gene sequences were identified using basic local alignment search tool (BLAST) with the nucleotide collection (nr/nt) database in the BLAST search program available at http://www.ncbi.nlm.nih.gov/.

### 3.3.4 Comparison of LAB profile between infant feces and breast milk

Healthy mother-infant (full-term) pair was chosen for this study (Table 11). Breast milk and infant fecal samples were taken from the first week until 10 months after birth. The breast milk samples were obtained by manual expression after cleaning nipples and areola by wiping with swab soaked in sterile water and discarding the first drops (Solis *et al*., 2010). The sample collected in a sterile plastic container was then immediately cooled and strictly maintained at 4 $^{o}$C throughout transportation period (not longer than 1 h). All fecal samples were kept frozen at -20 $^{o}$C. LAB diversity in all breast milk and fecal samples were analyzed by DGGE technique as described above.

### 3.3.5. Statistical analysis

The significant difference of LAB 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 $P<0.05$.

Table 10. The condition of infant involved in this study

| Infant codes | Gender | Delivery modes | Feeding types | Brand of milk powder | Duration of breast or formula milk feeding | Antibiotic use | Age at start to introduce solid food | Remarks |
|---|---|---|---|---|---|---|---|---|
| CF1-A | Male | Natural born | Combination milk | Hi-Q one plus brand (with FOS and GOS) | Combination feeding at the first day to 5 months of infant age | No | 4 months (porridge, pumpkin and banana) | - |
| CF2-C | Male | Cesarean born | Combination milk | Enfalac brand (with inulin and GOS) | Breast milk feeding at the first 2 day and combination feeding after 2 day toat the first day -5 months of infant age | Yes | 3 months (pumpkin, honey and carrot juice) | He was treat with green medicine (yaa kieow) and amoxicillin 125 mg, half of tea spoon 3 times/day for 1 week at 3 months of age |
| BF1-D | Female | Natural born | Breast milk | - | Breast milk feeding at the first week to 5 months | No | 4 months (banana) | - |
| BF2-E | Male | Cesarean born | Breast milk | - | Breast milk feeding at the first week to 5 months | No | 4 months (porridge and banana) | - |
| FF1-F | Male | Natural born | Formula milk | Hi-Q one plus brand (with FOS and GOS) Nan HA1 brand (hypoallergenic formula milk with *B. lactis* 0.00392%) | Combination milk feeding at the first 3 days and change to formula milk (Hi-Q one plus brand) at 1 week-2 months and Nan HA1 brand (2 months-5 months) of infant age | No | 4 months (porridge, banana and orange juice) | - |
| FF2-H | Male | Cesarean born | Formula milk | Enfalac brand (with inulin and GOS) | Combination milk feeding at the first 4 days and change to formula milk after 4 days to5 months of infant age | No | 4 months (banana, pumpkin and boiled egg york) | - |

Table 11. 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 | - | Breast milk feeding (first day to-10 months) |
| Antibiotic use | No | No |

## 3.4 Results

### 3.4.1 The conditions of each infant in this study

Six full term Thai healthy infants (Table 10) (one female: naturally born, breast fed infant (BF1-D) and five males: naturally born, combination fed infant (CF1-A), Cesarean born, combination fed infant (CF2-C), Cesarean born, breast fed infant (BF2-E), naturally born, formula fed infant (FF1-F) and Cesarean born, formula fed infant (FF1-H) have participated to the study. After birth, BF1-D and BF2-E infants received exclusive breast milk until 5 month. They were introduced solid food (banana and porridge) at 4 month of age. CF1-A infant received combination of breast milk and formula milk supplemented with fructo-oligosaccharide (FOS) and galacto-oligosaccharide (GOS) (Hi-Q one plus brand) and was introduced with porridge, pumpkin and banana as solid food at 4 months of age.CF1-C infant received exclusive breast milk at the first 2 days of age, there after changed to combination of breast milk and formula milk supplemented with inulin and GOS (Enfalac brand) and he was treated with amoxicillin and green medicine (Yaa kieow) for 7 days because he had exanthematous fever at 3 months of age. In addition, he was received the pumpkin juice, honey and carrot juice as a solid food at 3 months of age. FF1-F infant was received combination milk of breast and formula milk at 3 days after birth and exclusive formula

milk supplemented with FOS and GOS (Hi-Q one plus brand) for 2 months of age, there after changed to hypoallergenic formula milk supplemented with *Bifidobacterium lactic* 0.00392% (Nan HA1 brand) until 5 months of age and received porridge, banana and orange juice at 4 months of age. FF2-H infant received combination milk of breast and formula milk at 4 days of age and was fed with exclusive formula milk supplemented with inulin and GOS (Enfalac brand) for 5 months of age and received banana, pumpkin juice and boiled egg york at 4 months of age.

### 3.4.2 Population of LAB in infant feces

The fecal LAB population was determined from 6 healthy infants fed with breast milk (BF1-D infant and BF2-E infant), formula supplemented with FOS-GOS (FF1-F infant) and inulin-GOS (FF2-H infant) and a combination diet of breast milk and formula containing FOS-GOS (CF1-A infant) and inulin-GOS (CF2-C infant) (Figure 6). The combination feeding of breast milk and formula supplemented with FOS-GOS (CF1-A infant) and inulin-GOS (CF2-C infant) greatly enhanced LAB level. The number of LAB of two infants under the combination diet (CF1-A infant and CF2-C infant) was significantly higher than the exclusively breast fed (BF1-D infant and BF2-E infant) and formula infants (FF1-F infant and FF2-H infant) ($P<0.05$) at the first week after birth and remained stable at such levels ($10^9$ log CFU/g feces) for the first 5 months. Furthermore, the number of LAB of breast fed and formula supplemented with FOS-GOS fed infants slightly increased after first week and remained constant thereafter.

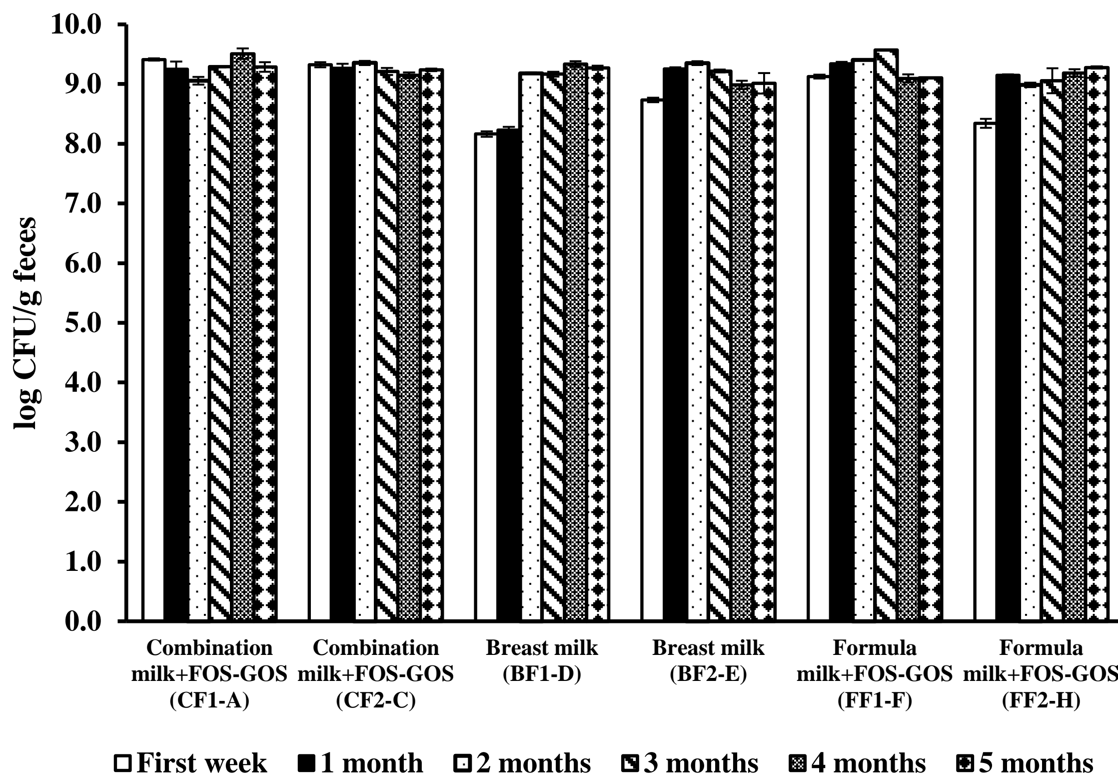


Figure 6. Colonization of LAB in fecal infants of combination diet mixing the breast milk and formula containing FOS-GOS (CF1-A infant) or combination diet mixing the breast milk and formula containing inulin-GOS (CF2-C infant), exclusive breast-fed diet (BF1-D infant) or, exclusive breast-fed diet (BF1-E infant), exclusive formula fed diet supplemented with FOS-GOS (FF1-F infant) or exclusive formula fed diet supplemented with inulin-GOS (FF2-H infant) from first week to 5 months.

### 3.4.3 Composition and diversity of fecal LAB from infants with different patterns of feedings

The nested PCR-DGGE profiles demonstrated that combination of breast milk and a formula supplemented with FOS-GOS (CF1-A infant) and inulin-GOS (CF2-C infant) and exclusive formula containing FOS-GOS (FF1-F infant) and inulin-GOS (FF2-H infant) distinctively enhanced the development of LAB (Figure 7. and Table 12). The number of band of LAB in infants fed with the combination diets of breast milk and a formula either containing FOS-GOS or inulin-GOS and both of exclusive formulae

containing FOS-GOS and inulin-GOS was not different when compared with the exclusively breast fed infant (BF2-E), except the number of LAB in exclusively breast fed infant (BF1-D) was lower than others as show in Table 13. Moreover, feeding type had no significant influence on the level of LAB species and diversity in 6 infants. Fourteen species of LAB were revealed in 6 infants. About 4-6 LAB species were dominantly present in each individual (Table 14 and Figure 8). Furthermore, the greatest number of LAB species was found in the infant with the exclusive breast fed infant (BF2-E infant) and exclusive formula fed diet supplemented with FOS-GOS (FF1-F infant). In addition, uncultured bacteria were commonly detected as the commensal intestinal microbiota in Thai infants, regardless feeding type. They were abundantly detected in feces from all infants. The combination feeding of breast milk and a formula supplemented with FOS-GOS and inulin-GOS harbored higher species levels of *L. plantarum* than others, while exclusive formula supplemented with FOS-GOS or inulin-GOS greatly promoted the growth of *L. gasseri* and *L. fermentum* In addition, exclusive breast milk feeding enhanced the growth of *L. crispatus* as show in Figure 8.

The combined feeding promoted the predominance of *L. plantarum* (band 1, 2, 3, 4) as well as *Leu. mesenteroides* (band 12). These particular strains were abundantly detected and remained persistently. Moreover, *L. gasseri* (band 30), *L. rhanosus* (band 32), *L. salivarius* (band 38) were predominant in the feces of the exclusively formula supplemented with FOS-GOS fed infant (FF1-F infant), while *L. gasseri* (band 33), *L. fermentum* (band 34), *L. paracasei* (band 44) were detected in the exclusively formula supplemented with inulin-GOS fed infant (FF2-H infant). In addition, the exclusively breast milk feeding promoted the abundance of *L. crispatus* (band 18), *L. gasseri*, (band 23) *L. helveticus* (band 26), *L. plantarum* (band 20, 21) as observed in the BF1-D and BF2-E infants as show in Figure 7 and Table 12.

The most noticeable feature of several LAB species appeared at the first week after birth and remained persistent thereafter was clearly demonstrated on the DGGE profiles. These included various strains of uncultured bacteria (band 22, 41), *L. plantarum* (band 1, 2, 3, 4), *Leu. mesenteroides* (band 12), *L. crispatus* (band 18), *L.*

*gasseri* (band 30, 33), *L. rhamnosus* (band 32) and *L. paracasei* (band 44). In addition, most of these LAB strain appeared in breast-fed infants and combination-fed infants. Thus, breast milk and prebiotic supplement had promoting effect on persistency of LAB in infants.

### 3.4.4 Comparison of LAB profiles between breast milk and infant feces

Breast milk samples and fecal samples from mothers and their exclusively breast fed infant were analyzed for LAB profile using nested PCR-DGGE technique. The LAB profile of breast milk was compared with the fecal LAB profile of infant fed on it (Figure 9 and Table 15). In the DGGE profile of breast milk, many uncultured bacteria (band 1, 2, 4, 8, 10, 11, 16), *Lactobacillus* sp. (band 3, 13, 14, 15), *L. plantarum* (band 5, 6), *L. gasseri* (band 7, 9), *Lactobacillus delbrueckii* (band 12), *L. rhamnosus* (band 17) and *L. casei* (band 18) were predominantly found. LAB species detected most frequently were *L. plantarum*, uncultured bacteria, *L. gasseri* and *L. delbrueckii* in all breast milk. Moreover, these species could also be found in the corresponding infant feces. This result indicated that breast milk was the important source of LAB developed in infant gut and therefore suggesting mother -infant transfer of LAB.

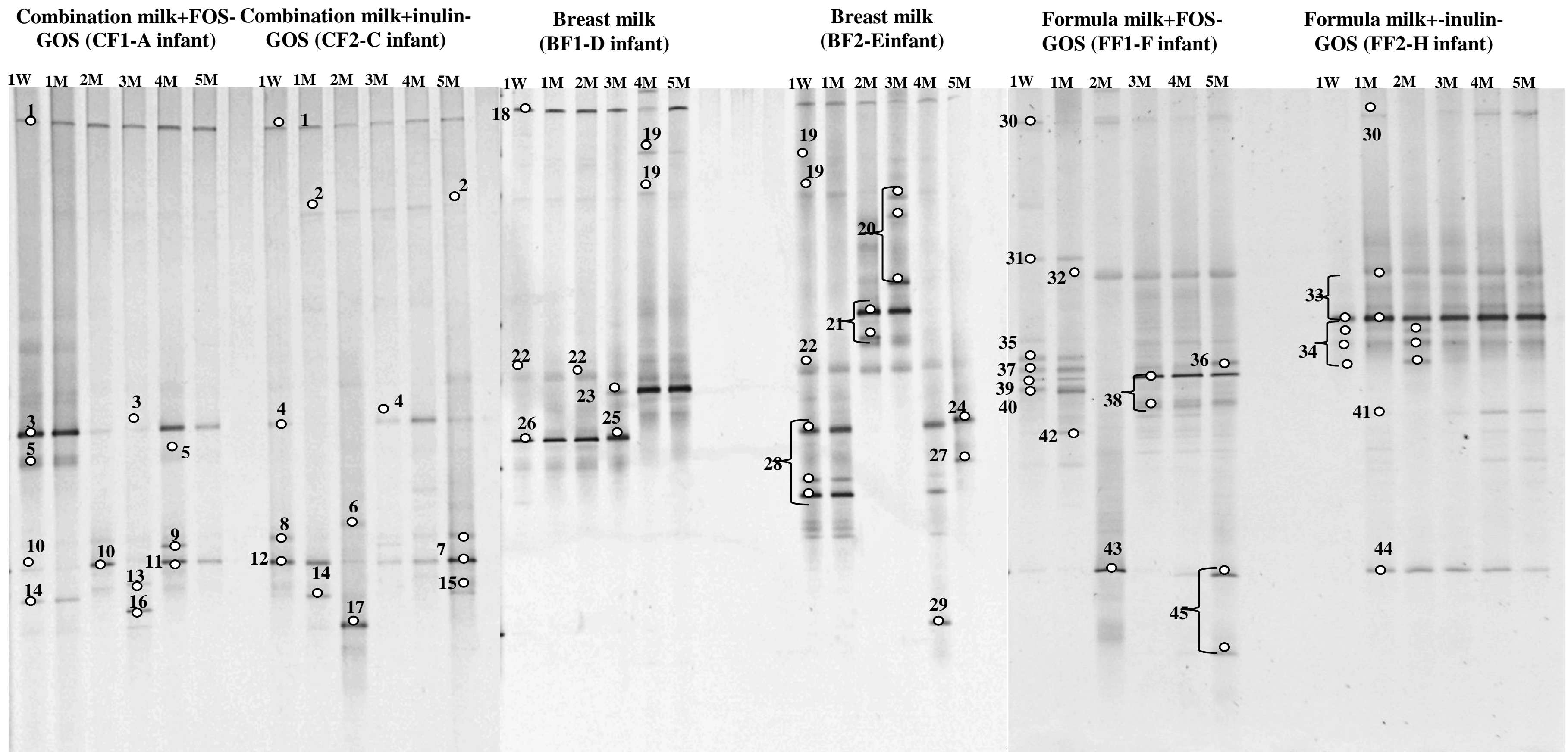


Figure 7. PCR-DGGE profiles representing the LAB diversity in combination diet of the breast milk and a formula containing FOS-GOS fed infant (CF1-A infant) and inulin-GOS fed infant (CF2-C infant), the exclusive breast-fed infant (BF1-D infant and BF1-E infant), the exclusive a formula supplemented with FOS-GOS fed infant (FF1-F infant) and inulin-GOS fed infant (FF2-H infant) from first week to 5 months after birth.

Table 12. Sequencing results of selected DGGE bands from the PCR-DGGE fingerprints of 6 infants

| Band no. | Closest relative | Accession number | Similarity (%) | Band no. | Closest relative | Accession number | Similarity (%) |
|---|---|---|---|---|---|---|---|
| 1 | *L. plantarum* | MF429768 | 100 | 24 | *L. fermentum* | MF429772.1 | 100 |
| 2 | *L. plantarum* | MG493269.1 | 100 | 25 | Uncultured bacteria | JF173113.1 | 100 |
| 3 | *L. plantarum* subsp. *plantarum* | LC311068.1 | 100 | 26 | *L. helveticus* | CP020029.1 | 100 |
| 4 | *L. plantarum* | MF429747.1 | 100 | 27 | *L. fermentum* | CP021790.1 | 100 |
| 5 | *L. plantarum* | CP022373.1 | 100 | 28 | Uncultured bacteria | JF164197.1 | 100 |
| 6 | *Leu. lactis* | LC269400.1 | 99 | 29 | *L. rhamnosus* | MF429170.1 | 100 |
| 7 | *Leu. lactis* | MF429764.1 | 99 | 30 | *L. gasseri* | KY969250.1 | 99 |
| 8 | Uncultured bacteria | KF103329.1 | 99 | 31 | Uncultured bacteria | JF109631.1 | 98 |
| 9 | *L. salivarius* | LT852760.1 | 99 | 32 | *L. rhanosus* | MF369976.1 | 99 |
| 10 | Uncultured bacteria | LT720554.1 | 99 | 33 | *L. gasseri* | KY969249.1 | 100 |
| 11 | Uncultured bacteria | MF225812.1 | 99 | 34 | *L. fermentum* | MF425520.1 | 100 |
| 12 | *Leu. mesenteroides* | MF357598.1 | 99 | 35 | Uncultured bacteria | JF109631.1 | 99 |
| 13 | Uncultured bacteria | LT721546.1 | 99 | 36 | *L. fermentum* | MF429767.1 | 98 |
| 14 | Uncultured bacteria | KF095264.1 | 100 | 37 | Uncultured bacteria | KF078774.1 | 99 |
| 15 | *L. casei* | MF429431.1 | 99 | 38 | *L. salivarius* | MF424441.1 | 100 |
| 16 | *Lactobacillus* sp. | KP178109.1 | 100 | 39 | Uncultured bacteria | HM281163.1 | 99 |
| 17 | *Leu. lactis* | KX507378.1 | 99 | 40 | Uncultured bacteria | KF088259.1 | 99 |
| 18 | *L. crispatus* | MF193438 | 100 | 41 | Uncultured bacteria | JF716114.1 | 93 |
| 19 | *Lactobacillus* sp. | KY937943.1 | 99 | 42 | Uncultured bacteria | GQ027577.1 | 100 |
| 20 | *L. plantarum* | MF429747.1 | 100 | 43 | *L. rhamnosus* | MF369917.1 | 100 |
| 21 | *L. plantarum* | MF429745.1 | 100 | 44 | *L. paracasei* | MF429031.1 | 100 |
| 22 | Uncultured bacteria | GQ101538.1 | 99 | 45 | *L. reuteri* | LT852761.1 | 100 |

Table 13. The number of visible DGGE bands of LAB from 6 healthy infants.

| Infant ages (months) | Combination milk + FOS-GOS (CF1-A) | Combination milk + inulin-GOS (CF2-C) | Breast milk (BF1-D) | Breast milk (BF2-E) | Formula milk + FOS-GOS (FF1-F) | Formula milk + inulin-GOS (FF2-H) |
|---|---|---|---|---|---|---|
| 1 week | 8 | 6 | 3 | 7 | 7 | 5 |
| 1 | 8 | 5 | 3 | 7 | 9 | 8 |
| 2 | 5 | 6 | 3 | 6 | 3 | 8 |
| 3 | 5 | 5 | 4 | 7 | 5 | 8 |
| 4 | 5 | 6 | 5 | 6 | 5 | 8 |
| 5 | 4 | 8 | 5 | 2 | 8 | 8 |
| Total | 35 | 36 | 23 | 35 | 37 | 45 |

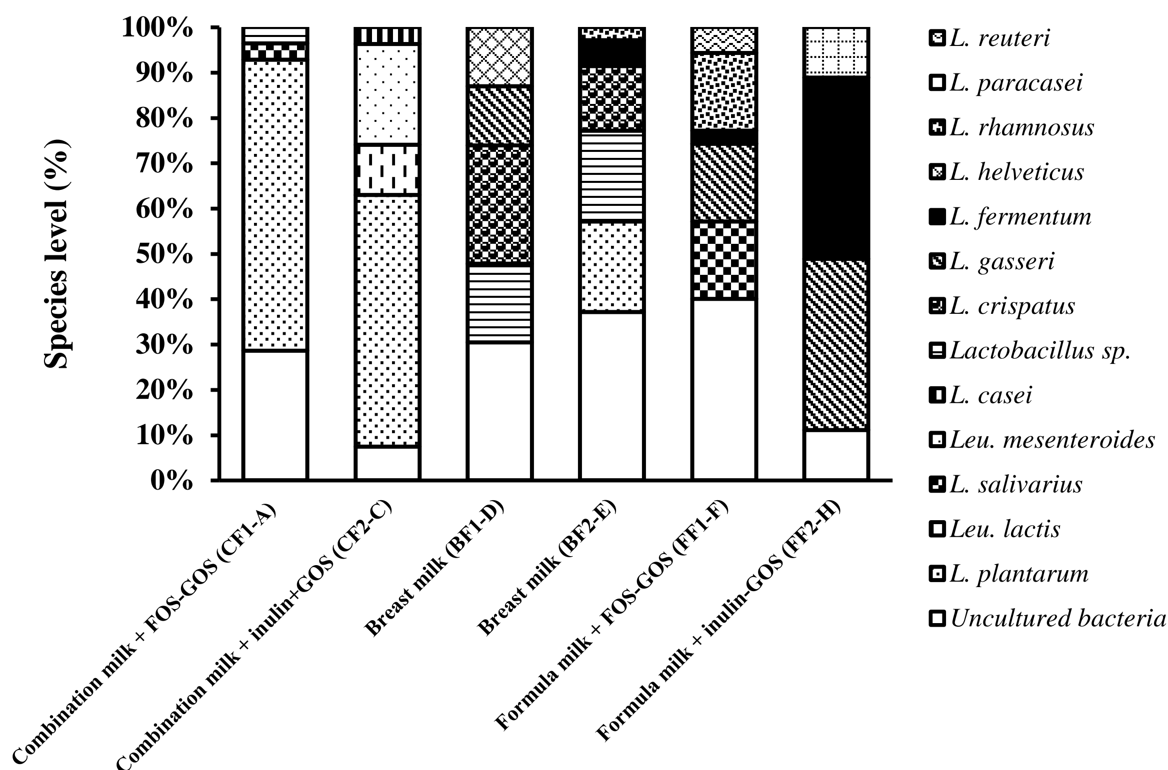


Figure 8. The compositions of LAB at the species level of combination of breast milk and a formula containing FOS-GOS fed infant (CF1-A infant) and inulin-GOS fed infant (CF2-C infant), the exclusive breast-fed infants (BF1-D infant and BF1-E infant), the exclusive formula supplemented with FOS-GOS fed infant (FF1-F infant) and inulin-GOS fed infant (FF2-H infant) from first week to 5 months after birth.

Table 14. The number of the cutting DGGE bands of each species and percentages of the species level in individual

| Strains | Infant CF1-A | | Infant CF2-C | | Infant BF1-D | | Infant BF2-E | | Infant FF1-F | | Infant FF2-H | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | The number of bands | *Species levels (%) | The number of bands | *Species levels (%) | The number of bands | *Species levels (%) | The number of bands | *Species levels (%) | The number of bands | *Species levels (%) | The number of bands | *Species levels (%) |
| Uncultured bacteria | 8 | 22.86 | 2 | 5.56 | 7 | 30.43 | 13 | 37.14 | 14 | 37.84 | 5 | 11.11 |
| *L. plantarum* | 18 | 51.43 | 15 | 41.67 | 0 | 0 | 7 | 20.00 | 0 | 0 | 0 | 0 |
| *Leu. lactis* | 0 | 0.00 | 3 | 8.33 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *L. salivarius* | 1 | 2.86 | 0 | 0 | 0 | 0 | 0 | 0 | 6 | 16.22 | 0 | 0 |
| *Leu. mesenteroides* | 0 | 0 | 6 | 16.67 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *L. casei* | 0 | 0 | 1 | 2.78 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Lactobacillus* sp. | 1 | 2.86 | 0 | 0 | 4 | 17.39 | 7 | 20.00 | 0 | 0 | 0 | 0 |
| *L. crispatus* | 0 | 0 | 0 | 0 | 6 | 26.09 | 5 | 14.29 | 0 | 0 | 0 | 0 |
| *L. gasseri* | 0 | 0 | 0 | 0 | 3 | 13.04 | 0 | 0 | 6 | 16.22 | 17 | 37.78 |
| *L. fermentum* | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 5.71 | 1 | 2.70 | 18 | 40 |
| *L. helveticus* | 0 | 0 | 0 | 0 | 3 | 13.04 | 0 | 0.00 | 0 | 0 | 0 | 0 |
| *L. rhamnosus* | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 2.86 | 6 | 16.22 | 0 | 0 |
| *L. paracasei* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 5 | 11.11 |
| *L. reuteri* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 5.41 | 0 | 0 |
| Total of the number of cutting band | 28 | | 27 | | 23 | | 35 | | 35 | | 45 | |
| Total of the number of visible bands | 35 | | 36 | | 23 | | 35 | | 37 | | 45 | |

* Specie level was determined using the following equation: A/B $\times$ 100 where A; the number of the bands of each species in individual and B; total of visible bands present in each infant.

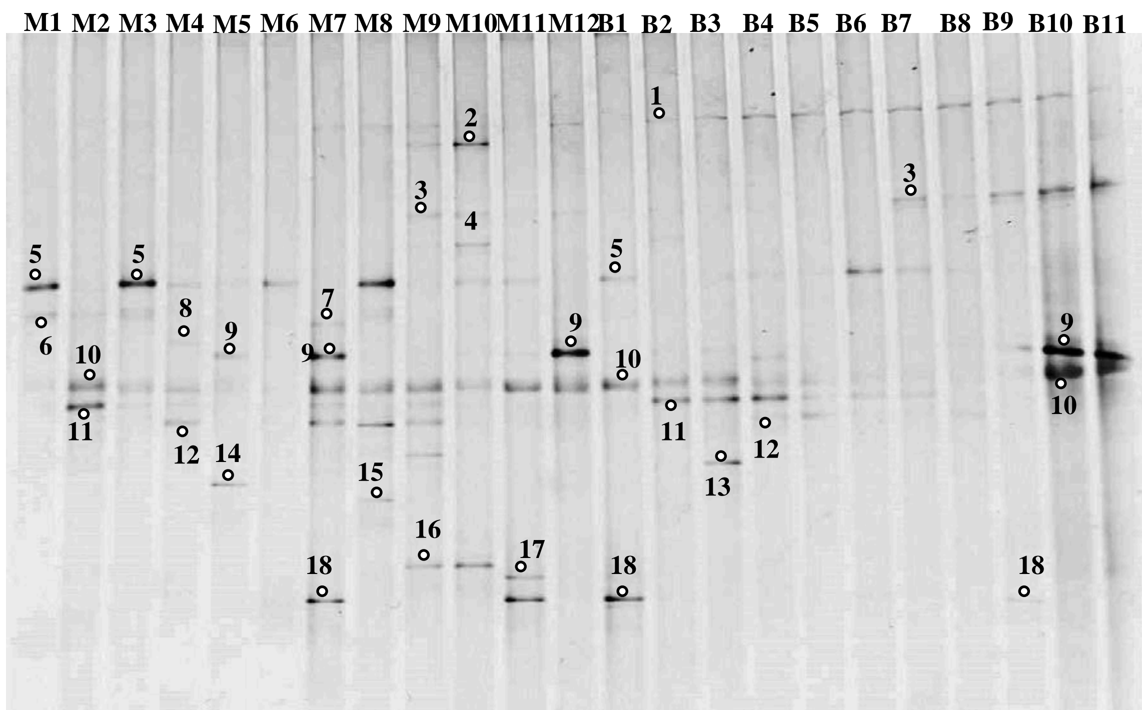


Figure 9. PCR-DGGE profiles of LAB communities in human milk samples from mother M1-M12 at first week to 11 months and fecal samples from her infant B1-B11 at first week to 10 months

Table 15. Sequencing results of selected DGGE bands from the PCR- DGGE of LAB in human milk samples from mother and fecal samples from her infant.

| **Band no.** | **Closest relative** | **Accession number** | **Similarity (%)** |
|---|---|---|---|
| 1 | Uncultured bacteria | KF103329.1 | 100 |
| 2 | Uncultured bacteria | JF212954.1 | 98 |
| 3 | Uncultured *Lactobacillus* sp. | KM250396.1 | 100 |
| 4 | Uncultured bacteria | KF101728.1 | 97 |
| 5 | *L. plantarum* | LC209103.1 | 100 |
| 6 | *L. plantarum* | LC208000.1 | 100 |
| 7 | *L. gasseri* | KY003103.1 | 99 |
| 8 | Uncultured bacteria | GQ137771.1 | 97 |
| 9 | *L. gasseri* | KY123806.1 | 100 |
| 10 | Uncultured bacteria | JF236696.1 | 99 |
| 11 | Uncultured bacteria | KF103329.1 | 100 |
| 12 | *L. delbrueckii* | CP018615.1 | 100 |
| 13 | Uncultured *Lactobacillus* sp. | KP777547 | 99 |
| 14 | *Lactobacillus* sp. | LC192790.1 | 99 |
| 15 | Uncultured *Lactobacillus* sp. | DQ857079 | 99 |
| 16 | Uncultured bacteria | KF101728.1 | 99 |
| 17 | *L. rhamnosus* | KU342056.1 | 100 |
| 18 | *L. casei* | JN182264.1 | 100 |

**3.5 Discussion**

The colonization of infant intestinal microbiota is influenced by many factors; type of feeding is one of the most important one which has a significant influence on the relative proportions of bacteria colonized in the infant gut (Guaraldi and Salvatori, 2012; Amenu, 2014; Thompson *et al*., 2015). Breast milk has been considered as the optimal diet for infant that contains a high level of complex human milk oligosaccharide (HMOs). These components function as natural prebiotic and selectively stimulate growth and diversity of beneficial bacteria such as bifidobacteria and LAB in the host gut (Satokari *et al*., 2002; Chen *et al*., 2007; Sela and Mills, 2010; Fernandez *et al*, 2013). Breast-fed infants are known to have a gastrointestinal flora that is dominated by bifidobacteria and LAB (Harmsen *et al*., 2000). This study showed that the combined diet of breast milk and a formula supplemented with either FOS-GOS or inulin-GOS greatly developed to higher number of fecal LAB than the breast milk and formula alone within the first week of life. Such high number remained stable up to 5 months of infant ages.

According to this study, the combined feeding between breast milk and formula milk supplemented with either inulin-GOS or FOS-GOS greatly enhanced LAB in correlating to formula milk supplemented with inulin-GOS or FOS-GOS confirmed by PCR-DGGE profile. The results confirmed the prebiotic roles of FOS-GOS or inulin-GOS may be helpful in the promotion of healthy microbiota to be proximity to that of breast-fed infants. Many considerable efforts have been made to mimic the composition of breast milk by the addition of different oligosaccharides to infant formula in order to induce breast-fed infant-similar microbiota colonization in formula-fed infants. FOS, GOS and inulin were often introduced to selectively stimulate growth of beneficial bacteria, such as bifidobacteria and LAB. Such approaches were employed with the intention to establish the healthy GI microbiota of formula-fed infants in the similar pattern of breast-fed ones (Haarman *et al*., 2006; Kapiki *et al*., 2007; Costalos *et al*., 2008; Roberfroid *et al*., 2010; Solis *et al*., 2010; Wang *et al*., 2015). Based on the analysis of human milk and high concentration of galactose, a mixture of inulin-GOS and

FOS-GOS were developed to mimic human milk for use in infant formulae (Boehm *et al*., 2002; Haarman and Knol, 2006). The composition of the intestinal microbiota is very complex. Numerous studies have demonstrated that mixture of FOS-GOS or inulin-GOS could stimulate LAB in similar pattern to milk oligosaccharides present in human milk (Moro *et al*., 2002; Ben *et al*., 2008).

In addition, breast milk also constitutes a source of commensal and probiotic bacteria which seems to play an important role in gut colonization and modulation of the infant gut. In recent years, analyses of the bacterial diversity of human milk have revealed that this biological fluid is an important source of live staphylococci, streptococci, bifidobacteria and LAB to the infant gut (Martin *et al*., 2003). LAB usually presents in the breast milk, such as *L. fermentum*, *L. plantarum*, *L. rhamnosus*, *L. helveticus*, *L. casei*, *L. gasseri*, *L. acidophilus* and *L. reuteri* (Soto *et al*., 2014). This study demonstrated that the LAB species most frequently found in breast milk were *L. plantarum*, uncultured bacteria, *L. gasseri* and *L. delbrueckii*. These species could relatively be detected in the fecal samples of the corresponding infant. This confirmed that breast milk was the important source of LAB. This also suggested the mother-to-infant transfer of LAB. The LAB transfer continued during the first months of life as supported by Heikkila and Saris (2003). The authors reported that infants would ingest between $1\times10^5$-$1\times10^7$ bacteria a day from consuming approximately 800 mL/day of breast milk. Moreover, breast milk from healthy women was reported to contain LAB approximately $2\times10^4$-$1\times10^5$ cfu/mL (Martin *et al*., 2003). LAB residing in maternal gut could possibly colonize mammary gland through an endogenous route (the so-called entero-mammary pathway), involving maternal dendritic cells and macrophages (Martin *et al*., 2004; Rodriguez, 2014). This study therefore strongly supported the transfer of indigineous LAB from the mothers to their babies. These LAB strains could be promoted and sustained in the infant gut depending upon prebiotic substrates either endogenously present in breast-milk or exogenously supplemented in infant formulae.

The diversity of LAB all of infants was not different, 4-6 LAB species were dominantly present in each individual and multiple strains of uncultured bacteria were

detected and abundant from all of infants. This result indicated that the combined feeding between breast milk and formula milk supplemented with either inulin-GOS or FOS-GOS and the exclusive feeding with formula supplemented with FOS-GOS or inulin-GOS were able to induce LAB that closely resembles LAB of exclusively breast-fed infant and also found in exclusively formula fed infant and combination fed infant.

Moreover, combination feeding was able to support the growth of *L. plantarum* and the exclusive feeding with formula supplemented with FOS-GOS and inulin-GOS enhance growth of *L. gasseri* and *L. fermentum* which was rather similar to that of exclusively breast fed. The mixtures of FOS-GOS or inulin-GOS were composed to mimic the molecule size distribution of human milk oligosaccharides to create optimal growth conditions for both bifidobacteria and lactobacilli (Boehm *et al*., 2002; Boehm *et al*., 2003. Moreover, addition of the specific prebiotic mixture of FOS-GOS or inulin-GOS results in a distribution of the different *Lactobacillus* species similar to that found in breast-fed infants (Haarman and Knol, 2006). Furthermore, the combined feeding promoted the predominance of *L. plantarum* as well as *Leu. mesenteroides*. These particular strains were abundantly detected and remained persistently. Moreover, *L. gasseri*, *L. rhanosus*, *L. salivarius* were predominant in the feces of the exclusively formula supplemented with FOS-GOS fed infant, while *L. gasseri*, *L. fermentum*, *L. paracasei* were detected in the exclusively formula supplemented with inulin-GOS fed infant. In addition, the exclusively breast milk feeding promoted the abundance of fecal *L. crispatus*, *L. gasseri*, *L. helveticus*, *L. plantarum.* Several studies demonstrated that LAB species composition appears 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 LAB in breast-fed infants and formula-fed infants (Satokari *et al*., 2002; Ahrne *et al*., 2005; Haarman and Knol, 2006; Salminen and Isolauri, 2006; Mitsou *et al*., 2008). However, there were different in the species levels of LAB in each individual, the combination feeding of breast milk and a formula supplemented with FOS-GOS and inulin-GOS harbored higher species levels of *L. plantarum* than other infants and *L. gasseri* and *L. fermentum* appeared to be enriched in formula fed infants

were fed with FOS-GOS or inulin-GOS. This result can be explained by the differential carbon source availability in the feeding. 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 (Bakker-Zierikzee *et al.,* 2005). Several strain of LAB such as *L. reuteri*, *L. rhamnosus*, *L. fermetum*, *L. gasseri*, *L. plantarum* greatly utilized GOS by producing ß-galactosidase to hydrolyze GOS (Mcbain and Macfarlane 2001; Schwab and Ganzle 2010; Sims *et al*., 2014). Multiple strains of *Lactobacillus* such as *L. plantarum, L. gasseri, L. rhamnosus* produce extracellular enzymes for FOS hydrolysis when exposed to FOS (Saulnier *et al.* 2007; Endo *et al.*, 2012). In addition, a minority of *Lactobacillus* was able to grow on inulin (Kaplan and Hutkins, 2000). 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. It has been demonstrated that *L. paracasei*, *L. plantarum* and *L. gasseri* fermented inulin as the sole energy sources (Makras *et al*. 2005; Takemura *et al*. 2010; Kanjan and Hongpattarakere 2017).

The persistent colonization by *Lactobacillus* is quite uncommon (Adlererth, 2008). Persistent colonization by a single strain of *Lactobacillus* over at 3 weeks was demonstrated in 17% of the infants in the first 6 months of life in Swedish infants (Ahrne *et al*., 2005). In contrast, our study demonstrated that many strain uncultured bacteria, *L. plantarum*, *Leu. Mesenteroides*, *L. crispatus, L. rhamnosus, L.fermentum, L. paracasei* and *L. gasseri* appeared at the first week after birth and there were persistent thereafter. These persistent strains were likely true colonizers of the infant gut. Likewise, Timmerman *et al*. (2017) reported that the genus of *Lactobacillus* prolonged colonization and persistence in GI tract of infants. Moreover, the most of these strains appeared in combination fed infants and exclusively formula fed infants. This result could suggest that addition of the mixture of FOS-GOS or inulin-GOS has promoting effect on growth and persistency of LAB in infants.

**3.6 Conclusion**

The number of fecal LAB from the infants fed with the combination diet of breast milk and a formula containing FOS-GOS (CF1-A) and inulin-GOS (CF2-C) was higher than that from both exclusively breast-fed (BF1-D and BF2-E) and exclusively formula fed ones. The number of DGGE bands of LAB in infants with combination (CF1-A infant and CF2-C infant) and exclusive formula feeding (FF1-F infant and FF2-H infant) and the exclusively breast-fed infants (BF2-E) was not different, while the number of LAB in exclusively breast fed infant (BF1-D) was lower than another. 14 species of LAB were found from 6 infants and each infant presented 4-6 species of LAB and the most number of LAB species was found in the infant with the exclusive breast fed infant (BF2-E infant) and exclusive formula fed diet supplemented with FOS-GOS (FF1-F infant). Moreover, uncultured bacteria were commonly found in the commensal intestinal microbiota in Thai infants. Combination feeding was able to support the growth of *L. plantarum* and *Leu. mesenteroides.* The exclusive feeding with formula supplemented with FOS-GOS enhance growth of *L. rhamnosus* and *L. gasseri*, while the formula supplemented with inulin-GOS sustained growth of *L. gasseri*, *L. fermentum* and *L. paracasei.* In addition, exclusive breast milk feeding was capable to promote the growth of *L. gasseri*, *L. crispatus*, *L. plantarum* and *L. helveticus*.

# CHAPTER 4

# MECHANISMS OF CHOLESTEROL REMOVAL BY LACTIC ACID BACTERIA (LAB) AND BIFIDOBACTERIA ISOLATED FROM BREAST MILK AND INFANT FECES

## 4.1 Abstract

Thirty strains of lactic acid bacteria (LAB) and four strains of bifidobacteria isolated from healthy infant feces and breast milk were evaluated for cholesterol-lowering activity through various mechanisms. *Enterococcus feacalis* (10), *Enterococcus feacium* (6), *Lactobacillus plantarum* (7), *Lactobacillus casei* (2), *Lactobacillus rhamnosu*s (4), *Lactobacillus paracasei* (1), *Bifidobacterium longum* (2) and *Bifidobacterium bifidum* (2) were determined for the ability to assimilate cholesterol from growth media, bile salt hydrolase activity and cholesterol binding property. All of 34 strains were able to assimilate cholesterol in the 0.3 % oxgall bile containing medium in the range of 14.39-65.57 µg/ml using *O*-phthalaldehyde method. *L. plantarum* 108 displayed the highest assimilation. The bile salt hydrolase (BSH) activity was only confined among nine strains of *Ent. faecalis* and five strains of *Ent. faecium* by showing precipitation zone on MRS agar supplemented with 0.5% (w/v) sodium salt of taurodeoxycholic acid (TDCA). The cholesterol binding ability of the resting cells (live cells) and dead cells (heat-killed cells) was exhibited within all LAB and bifidobacteria range of 7.62-19.49 mg/g dry weight and 3.48-13.65 mg/g dry cell weight. Furthermore, *Ent. faecium* EMA410.4, *Ent. faecalis* M134 and *Ent. faecalis* EM17.3 strongly adhered to Caco-2 and HT-29 cell lines. According to this study, *Ent. faecium* EMA410.4, *Ent. faecalis* M134 and *Ent. faecalis* EM17.3 had greatly ability to produce BSH enzyme, remove cholesterol from the media during growth, remove cholesterol *via* binding to cellular surface of resting and dead cell and strongly adhered to Caco-2 and HT-29 cell lines. Therefore, these strains can be potentially applied as a good probiotic candidate for lowering cholesterol level.

## 4.2 Introduction

Cholesterol is a soft waxy substance produced in the liver. It is a natural component of the fats in the bloodstream and in all cells of the body (Ajmal and Ahmed, 2009). It is an important part of a healthy body because it is used to form cell membranes, certain hormones, synthesize vitamin D and form bile secretions necessary for digestion. However, the high level of serum cholesterol in the blood is known as hypercholesterolemia, the major cause of cardiovascular disease which is the leading cause of one-third of all deaths worldwide (Law *et al*., 1994; Ma, 2006; Tok and Aslim, 2010). Therefore, decreasing serum cholesterol level is very important for preventing the cardiovascular diseases. Using of the cholesterol-lowering drugs has been associated with many adverse effects that limit treatment compliance as well as quality of life (Golomb and Evans, 2008). Therefore, there is growing interest in the preventing and lowering hypercholesterolemia by practicing dietary control or using prebiotic and probiotic has emerged recently (Helim *et al*., 2016).

Probiotic is defined as living microorganisms, which can elicit positive influence on host health by improving its intestinal microbial balance when a sufficient number was administered with high survival in the intestinal ecosystem (Fuller, 1989; Gismondo *et al*., 1999). Other health benefits have also been reported upon their daily consumption of probiotics, including reducing blood pressure (Lye *et al*., 2009; Kechagia *et al*., 2013) and reducing cholesterol level (Lye *et al*., 2010; Homayouni *et al*., 2012; Ichim *et al*., 2016). The most common probiotic bacteria that possess health benefits include *Lactobacillus* spp., *Bifidobacterium* spp. and *Enterococcus* spp. (Scheinbach, 1998; Fijan, 2014; Varankovich *et al*., 2015; Linares *et al*., 2017). These bacteria have a major role to play in the cholesterol lowering mechanism base of on the relevant characteristics including enzymatic deconjugation of bile acid by bile salt hydrolase (BSH), co-precipitation of cholesterol with free bile acids, binding cholesterol to surface, or assimilation of cholesterol into the cellular membrane or cytoplasm (Tanaka *et al*., 2000; Ahn *et al*., 2003; Lye *et al*., 2010; Tomara-Duchesneau *et al*., 2014). Bile salt hydrolase

(BSH; cholylglycine hydrolase; EC3.5.1.24), that catalyzes the hydrolysis of glycine-and taurine-conjugated bile salts into amino acid residues and free bile salts (bile acids) in the enterohepatic circulation. Cholesterol is the precursor of primary bile salts that are synthesized in the liver and are stored and concentrated as conjugated bile salts in the gall bladder. Conjugated bile salts are released into the small intestine for absorption of dietary fat, hydrophobic vitamins and other fat-soluble compounds (Liong and Shah, 2005; Begley *et al*., 2006). Once deconjugated, bile acids are less soluble and absorbed by the intestines, leading to higher excretion into the feces. Replacement of new bile salts from cholesterol as a precursor, resulting in lowering of serum cholesterol (Ooi and Liong, 2010). BSH activity has been widely detected in GI species of the genera *Lactobacillus* spp., *Bifidobacterium* spp. and *Enterococcus* spp. (Franz *et al*., 2001; Moser and Savage, 2001; Knarreborg *et al*., 2002; Wijaya *et al*., 2004; Parvez *et al*., 2006; Shehata *et al*., 2016). Cholesterol was also removed by *Lactobacillus*, *Bifidobacterium* and *Enterococus* by up-take or assimilation into the cellular membranes during growth, thus making it unavailable for absorption from the intestines into the blood (Walker and Gilliland, 1993; Tok and Aslim, 2010; Tomara-Duchesneau *et al*., 2014; Guo *et al*., 2016). Furthermore, heat-killed cells and resting cells of some probiotic stains can remove cholesterol from the media due to cholesterol attachment to the cellular membrane (Kimoto *et al*., 2002; Lye *et al*., 2010). Thus, it appears that some probiotic strains can remove cholesterol from media both by binding of cholesterol to dead and resting cells. In addition, some previous studies performed in vitro have suggested that the effect of cholesterol lowering from the combination of multiple mechanisms including cholesterol assimilation during bacterial growth, cholesterol binding to cell membrane and deconjugation of bile salts (Liong, and Shah, 2005; Lye *et al*., 2010).

*Enterococcus* is a group of LAB that is typically present in human GI (Fisher and Phillips, 2009; Montalto *et al*., 2009; Duerkop *et al*., 2012). Several species of enterococci are known to be opportunistic pathogens and major causes of nosocomial infections causing human diseases, such as bacteriamia, endocarditis or urinary tract infection (Kayser, 2008; Franz *et al*., 2011). Such pathogenic strains often carry multiple

antibiotic resistance and virulence factors (Araujo and Ferreira, 2013). Therefore, safety assessments of enterococci with special attention paid to antibiotic resistance, virulence traits and hemolytic activity are the essential elements in selection of the probiotic strains (Nueno-Palop and Narbad, 2011; Aslam *et al*., 2012). However, *Enterococcus* has been the target of several studies for probiotics (Lund *et al*., 2000; Zeyner and Boldt, 2006; Nueno-Palop and Narbad, 2011; Cebrian *et al*., 2012; Fijan, 2014). The genera comprises more than 20 species, but *Enterococcus faecalis* and *Enterococus faecium* were frequently found both in food and in clinical samples (Gomes *et al*., 2008; Araujo and Ferreira, 2013). Some *Enterococus* strains were able to survive after passing through condition simulated upper part of human gastrointestinal tract with high survival rate (Faye *et al*., 2012; Barbosa *et al*., 2014 Santos *et al*., 2015). Some produced antimicrobial compounds such as enterocin ( De Vuyst *et al*., 2003; Khan *et al*., 2010; Rehaiem *et al*., 2016). In addition, *Enterococcus* spp. could reduce cholesterol level includes enzymatic deconjugation of bile acid by bile salt hydrolase, binding cholesterol to surface, or assimilation of cholesterol into the cellular membrane (Wijaya *et al*., 2004; Santos *et al*., 2015; Guo *et al*., 2016).

The ability to adhere is a prerequisite for bacterial colonization (Klaenhammer and Kullen, 1999). Adhesion of probiotic to the intestinal epithelium is also considered important for modulation of the immune system (Isolauri *et al*., 2001), balance of intestinal microflora, intestinal bacterial enzyme activity and stabilization of intestinal permeability (Walker and Duffy, 1998). Therefore, the ability of adherence of bacterial strain to intestinal epithelium is considered as an important selection criterion for LAB and bifidobacteria intended for probiotic use. Caco-2 and HT-29 cell lines isolated from human colonic adenocarcinoma have been most widely used models to assess the adhesion ability of probiotic strains *in vitro* as they closely mimick the normal intestinal epithelium (Gagnon *et al*., 2013; Sharma and Kanwar, 2017). Several studies have been conducted using HT-29 and Caco-2 to screen the adhesion of probiotic strains (Laparra and Sanz, 2009; Martin *et al*., 2011; Gagnon *et al*., 2013; Sharma and Kanwar, 2017). In the present work, LAB and bifidobacteria strains from infant feces and breast milk were

screened based on cholesterol-lowering mechanisms such as bile salt hydrolase (BSH) activity, cholesterol assimilation and cholesterol removal via binding to cellular surface as well as examining adhesion ability of LAB to HT-29 and Caco-2.

## 4.3 Materials and Methods

### 4.3.1 Microorganisms and cultivation condition

A total of 34 isolates of LAB and bifidobacteria were collected from food biotechnology laboratory, Department of Industrial Biotechnology, Faculty of Agro-Industry, Prince of Songkla University. 16 isolates of *Enterococcus* were isolated from breast milk and 14 isolates of *Lactobacillus* and 4 isolates of *Bifidobacterium* were isolated infant feces, *Enterococcus feacalis* (10), *Enterococcus feacium* (6), *Lactobacillus plantarum* (7), *Lactobacillus casei* (2), *Lactobacillus rhamnosus* (4), *Lactobacillus paracasei* (1), *Bifidobacterium longum* (2) and *Bifidobacterium bifidum* (2) (Table 16). These strains were able to survive after passing through condition simulated upper part of human gastrointestinal tract with high survival rate. In addition, they inhibited many food-borne pathogenic bacteria and strongly adhered to mucin. Each stock culture was stored at -20°C in 35% glycerol (v/v). LAB and bifidobacteria were subcultured three times in sterile de Mann, Rogosa, Sharpe (MRS) broth (Himedia, Mumbai, India) and using 10% inoculum and incubated at 37°C for 24 h under anaerobic condition before experimental use.

### 4.3.2 Screening of cultures for bile salt hydrolase (BSH) activity

The ability of strains to deconjugate bile salt was determined according to Dong *et al*. (2012). LAB and bifidobacteria were cultivated in MRS broth supplemented with 0.15% L-cysteine (Sigma-Aldrich, Steinheim, Germany) at 37 °C for 24 h. Overnight cultures (10 µl) were spotted on MRS agar plate supplemented with 0.5% (w/v) sodium salt of taurodeoxycholic acid (TDCA) (Merck, Darmstadt, Germany) and 0.37 g/l of $CaCl_2$ (Merck, Darmstadt, Germany). The plates were incubated anaerobically at $37^{o}C$ for 72 h. MRS agar plates without TDCA were used as controls. The precipitation zone

surrounding colonies (opaque halo) or the formation of opaque granular white colonies with silvery shine was considered as positive reaction.

### 4.3.3 Cholesterol reduction

Cholesterol reduction was studied according to Lye *et al*. (2010) with some modification. Thirty strains of LAB and four strains of bifidobacteria grown at 37$^{o}$C in MRS broth supplemented with 0.15% L-cysteine and 100 µg/ml of alcohol-soluble cholesterol (Himedia, Mumbai, India) were inoculated in different media containing 0% and 0.3% w/v of oxgall (Merck, Darmstadt, Germany) as a bile source and incubated for 24 h at 37 $^{o}$C. After the incubation period, cells were removed from the broth by centrifugation at 8,100×g for 10 min at 4 $^{o}$C. The total amount of cholesterol in the spent broth and un-inoculated sterile broth (negative control) were determined by a modified colorimetric method as described by Rudel and Morris. (1973). One milliliter ml of supernatant, 2 ml of 50% KOH (Merck, Darmstadt, Germany) and 3 ml of 95% ethanol were mixed and then heated for 10 min in a 60 °C water bath. After cooling, 5 ml of hexane was added, followed by mixing and addition of 1 ml of distilled water and tubes were allowed to stand at room temperature to permit phase separation. A sample of 2 ml aliquot of hexane phase was transferred to a clean tube and the hexane phase was then evaporated under the flow of nitrogen gas. A 4 ml of freshly prepare *O*-phthalaldehyde (Merck, Darmstadt, Germany) reagent was added to each tube and allowed to stand at room temperature for 10 min. Following the addition of 2 ml of concentrated sulfuric acid and standing for an additional 10 min, the absorbance at 550 nm was read against a reagent blank. Absorbance values were compared to those obtained with cholesterol standards. Finally, the percentage cholesterol reduction was estimated as follows:

Cholesterol reduction (%) = [100 – (cholesterol (µg) in the supernatant of the inoculated MRS broth/ cholesterol (µg) in the supernatant of MRS broth without inoculation) ×100 (Kim *et al.,* 2008).

Table 16. Origin of LAB and bifidobacteria strains

| **Bacterial strains** | **Origin** |
|---|---|
| *L. paracasei* I321 | Infant feces |
| *L. rhamnosus* A1 | Infant feces |
| *L. rhamnosus* A2 | Infant feces |
| *L. rhamnosus* A9 | Infant feces |
| *L. rhamnosus* A10 | Infant feces |
| *L. casei* A27 | Infant feces |
| *L. casei* AN33 | Infant feces |
| *L. plantarum* 108 | Infant feces |
| *L. plantarum* A121 | Infant feces |
| *L. plantarum* A123 | Infant feces |
| *L. plantarum* 124 | Infant feces |
| *L. plantarum* 178 | Infant feces |
| *L. plantarum* 184 | Infant feces |
| *L plantarum* I61 | Infant feces |
| *Ent. faecalis* M118 | Breast milk |
| *Ent. faecalis* M134 | Breast milk |
| *Ent. faecalis* M237 | Breast milk |
| *Ent. faecalis* M125 | Breast milk |
| *Ent. faecalis* M114 | Breast milk |
| *Ent. faecalis* M135 | Breast milk |
| *Ent. faecalis* M117 | Breast milk |
| *Ent. faecalis* M145 | Breast milk |
| *Ent. faecalis* M126 | Breast milk |
| *Ent. faecalis* EM17.3 | Breast milk |
| *Ent. faecium* EMA32.1 | Breast milk |
| *Ent. faecium* EMA47.2 | Breast milk |
| *Ent. faecium* EMA49.5 | Breast milk |
| *Ent. faecium* EMA31.6 | Breast milk |
| *Ent. faecium* EMA410.4 | Breast milk |
| *Ent. faecium* EMA31.8 | Breast milk |
| *B. longum* AN37 | Infant feces |
| *B. longum* AN38 | Infant feces |
| *B. bifidum* AN158 | Infant feces |
| *B. bifidum* AN161 | Infant feces |

### 4.3.4 Cholesterol removal via binding to cellular surface

The ability of non-growing cell to remove cholesterol (heat-killed cells and resting cells) was determined according to a modified method of Tok and Aslim. (2010). Freshly prepared MRS broth containing 0.15% L-cysteine was inoculated with each LAB and bifidobacteria strain and incubated for 24 h at 37 ºC. After incubation, the cells were harvested by centrifugation at 8,100×g, for 10 min at 4 °C and washed twice with sterile distilled water. In the case of the heat-killed cells, the cell pellet was suspended in 10 ml of saline and autoclaved at 121 °C for 15 min. The heat-killed cells were then suspended in MRS broth supplemented with 0.3% (w/v) oxgall and 100 µg/ml of cholesterol. To prepare resting cells, the cell pellet was suspended in 10 ml of sterile phosphate buffer (0.05 M, pH 6.8) containing 0.3% (w/v) oxgall and 100 µg/ml of cholesterol. All strains were incubated at 37 °C for 24 h. Upon fermentation/incubation, the mixture was centrifuged (8,100×g 4°C, 10 min) and the remaining cholesterol concentration in the broth was measured using the method described above. Reduction of cholesterol by dead cell, and resting cells was expressed in dry weight (Lye *et al*., 2010). The following equation was used:

$$\text{Cholesterol reduction} = (C1 - C2) / (W2)$$

where C1 and C2 were the amount of cholesterol present in the fermentation broths at time = 0 and time = 24 h, respectively, and W2 was the dry weight of the individual culture at time = 24 h.

### 4.3.5 Scanning electron microscopy (SEM)

The attachment of cholesterol onto LAB cells was observed through SEM. Resting cells and dead cells were prepared in section 4.3.4. Both cells were immersed in fixative solution (2.5% glutaraldehyde in 0.1 M phosphate-buffer) at room temperature for 2 h. The fixative was rinsed with 0.1 M phosphate-buffer three times. The pellet was then postfixed with 1% (w/v) osmium tetroxide in 0.1 M phosphate-buffer. After that, the pellet was washed twice with 0.1 M phosphate-buffer and dehydrated with an ethanol series (50%, 60% 70%, 80%, 90% and 100%) for 2 min in each concentration (Choi and

Chang, 2015). The dehydrated specimens were dried in a critical point drier (CPD) with liquid carbon dioxide and sputter coated with gold-palladium. Cell morphology was observed with a FEI Quanta400 FEG Scanning Electron Microscope (FEI Company, Hillsboro, Oregon, USA) at 40,000x magnification and operating at 20 kV of accelerating voltage.

### 4.3.6 Adhesion of LAB to epithelium cell

The Caco-2 cell and HT-29 were routinely maintained in tissue culture flask in cell culture medium, Dulbecco's modified Eagle medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FCS), 1% (v/v) non-essential amino acids (NEAA), and 1% (v/v) penicillin-streptomycin solution, respectively. The cell cultures were incubated at 37 ºC with 5% $CO_2$ and 90% relative humidity to achieve. The culture medium was replaced every 2 days. When the cell reach the subculturing density of 50% confluence, the medium was removed from tissue culture flask and the cell cultures were added with 2 ml of Trypsin-EDTA solution and incubated for 37 ºC for 5 min and then, wash the cell cultures with culture medium containing 10% FCS. The cell suspension was centrifuged at 500×g for 3 min. After removing supernatant, the cell pellet was resuspended in the cell culture medium and seeded in 24 well culture plates at the density of $2\times10^5$ cells/well and incubated at 37 ºC with 5% $CO_2$ and 90% relative humidity to achieve. The culture medium was changed every 2 days and 24 h before an adhesion assay. The cell cultures were used for the adhesion assays after 18 days of incubation and complete differentiation (Gleinser *et al*., 2012).

The late log phase of LAB was centrifuged at 2,000×g for 10 min and the bacterial cells were washed once in phosphate buffered saline (PBS; pH 7.4). The bacterial cell $10^8$ CFU/ml was suspended in DMEM without antibiotic and FCS and 100 µl of bacterial suspension ($N_{Initial}$) were added to each well. After incubation for 1 h at 37 ºC in 5% $CO_2$ and 90% relative humidity, non-adherent bacteria was removed by washing each well three times with 1 ml of PBS. Cell monolayers were then lysed by the addition 1 ml of ice-cold double-distilled $H_2O$ ($N_{Adhere}$) and serial ten-fold dilutions in

PBS were plated in spots of 10 µl on MRS agar plates to enumerate of adherent bacteria (Gleinser *et al*., 2012). *B. bifidum* S17, which was previously described as adherent strain, was used as positive control. The results were expressed as the adhesion percentage with respect to the initial bacterial inoculum. The percentage of adhesion was calculated according to the following equation:

$$\% \text{ Adhesion} = (\text{Log CFU } N_{\text{Adhere}}/\text{Log CFU } N_{\text{Initial}})\times 100$$ (Uraipan *et al*., 2014).

### 4.3.7 Statistical analysis

All determinations were performed in triplicate. The data were subjected to analysis of variance (ANOVA) and significant differences among mean values were determined by SPSS 17.0. The values were expressed as means ± SD. Statistical significance was evaluated using Duncan's Multiple Range Test. Statistical significance was accepted at $P < 0.05$.

## 4.4 Results

### 4.4.1 Bile salt hydrolase (BSH) activity

All LAB and bifidobacteria strains were screened for BSH activity by spot on MRS agar supplemented with 0.5% (w/v) TDCA. Among 9 strains of *Ent. faecalis* and 5 strains of *Ent. faecium* from breast milk were able to hydrolyze TDCA to precipitate out deoxycholate by halos of the precipitates zone around active colonies on the MRS plate (Figure 10). The precipitation zone ranging from 1.17 to 3.30 mm and *Ent. faecalis* M317 exhibited the highest precipitation zone (Table 17).

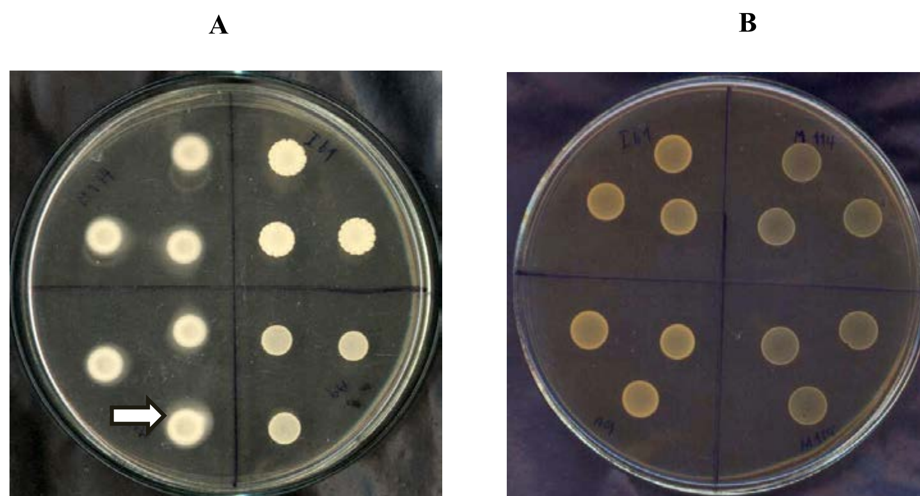


Figure 10. Bile salt hydrolase (BSH) activity of LAB as detected by the qualitative agar plate assay. A, MRS supplemented with 0.5% TDCA (sodium salt of taurodeoxycholic acid) and 0.37% g/l $CaCl_2$; B, MRS as control; Bile-salt hydrolysis positive was represented by white arrow.

Table 17. Bile-salt-hydrolyzing activity as detected by the qualitative agar plate assay

| **Bacterial strains** | **Diameter of the precipitation zone (mm)** |
|---|---|
| *L. paracasei* I321 | 0 |
| *L. rhamnosus* A1 | 0 |
| *L. rhamnosus* A2 | 0 |
| *L. rhamnosus* A9 | 0 |
| *L. rhamnosus* A10 | 0 |
| *L. casei* A27 | 0 |
| *L.casei* AN33 | 0 |
| *L. plantarum* 108 | 0 |
| *L. plantarum* 178 | 0 |
| *L. plantarum* A121 | 0 |
| *L.plantarum* A123 | 0 |
| *L. plantarum* 124 | 0 |
| *L. plantarum* I61 | 0 |
| *L.plantarum* 184 | 0 |
| *Ent. faecalis* M118 | $2.67 \pm 0.31^{B}$ |
| *Ent. faecalis* M134 | $2.60 \pm 0.31^{B}$ |
| *Ent. faecalis* M237 | 0 |
| *Ent. faecalis* M125 | $2.50 \pm 0.20^{BC}$ |
| *Ent. faecalis* M114 | $2.60 \pm 0.53^{B}$ |
| *Ent. faecalis* M135 | $1.90 \pm 0.26^{CD}$ |
| *Ent. faecalis* M117 | $3.30 \pm 0.36^{A}$ |
| *Ent. faecalis* M145 | $2.63 \pm 0.15^{B}$ |
| *Ent. faecalis* M126 | $1.40 \pm 0.35^{EF}$ |
| *Ent. faecium* EMA32.1 | $2.17 \pm 0.15^{BC}$ |
| *Ent. faecalis* EM17.3 | $1.17 \pm 0.57^{F}$ |
| *Ent. faecium* EMA47.2 | 0 |
| *Ent. faecium* EMA49.5 | $1.47 \pm 0.50^{DE}$ |
| *Ent. faecium* EMA31.6 | $1.97 \pm 0.45^{CD}$ |
| *Ent. faecium* EMA410.4 | $1.3 \pm 0.21^{EF}$ |
| *Ent. faecium* EMA31.8 | $2.30 \pm 0.20^{BC}$ |
| *B. longum* AN37 | 0 |
| *B. longum* AN38 | 0 |
| *B. bifidum* AN158 | 0 |
| *B. bifidum* AN161 | 0 |

All data mean values of triple determination ± standard deviation (SD). Different superscript uppercase letters (A-F) indicate significant difference ($P < 0.05$).

### 4.4.2 Removal of cholesterol from broth by LAB and bifidobacteria during growth

The removal of cholesterol by 30 strains of LAB and 4 strains of bifidobacteria in MRS broth supplemented with 0.3% oxgall compared with MRS broth without 0.3% bile salt (oxgall) during 24 h at 37 °C under anaerobic condition (Table 18). All probiotic bacterial strains were able to reduce cholesterol at varying degrees. Analysis of variance showed that the percentage of cholesterol reduction varied significantly ($P<0.05$) among different strains. Cholesterol reduction of LAB and bifidobacteria in the medium containing 0.3% bile salt was higher than the medium without bile salt. The cholesterol reduction ranged 5.69-27.20% and 14.39-65.57% in the MRS broth medium containing 0% and 0.3% bile salt, respectively. In particular, LAB strains *E. faecium* EMA31.6, *E. faecium* EMA49.5, *E. faecium* EMA47.2, *E. faecium* EMA410.4 and *L. plantarum* 108 showed significantly ($P<0.05$) higher cholesterol removal ability (58.37%, 58.87%, 58.96, 61.11 and 65.57, respectively) in the MRS broth medium supplemented with 0.3% bile salt compared with the other strains.

### 4.4.3 Cholesterol binding ability of LAB and bifidobacteria on the cell surface

The cholesterol binding ability was tested with resting cells (live cells suspended in phosphate buffer) and dead cells (heat-killed). The ability of 30 strains of LAB and 4 strains of bifidobacteria, either resting cell or dead cell to remove cholesterol was assessed (Table 19). The removal of cholesterol varied significantly ($P < 0.05$) among resting cell and dead cells, ranging 7.62-19.49 mg/g dry cell weight and 3.48-13.65 mg/g dry cell weight, respectively. The strain of *Ent. faecalis* M145 achieved the highest cholesterol removal by resting cell and *Ent. faecalis* M134 exhibited the highest cholesterol removal by dead cell. In addition, all strains exhibited higher cholesterol removal when cells were resting compared with and dead cells. Moreover, the binding of cholesterol onto bacterial cell was observed using SEM (Figure 11). The bounded cholesterol particle on the cell surface resulted in the roughness of cell wall. Large amounts of cholesterol adhered on the cell surface of resting cell. On the contrary, small amounts of cholesterol adhered on the cell surface of dead cell.

Table 18. Cholesterol reduction by LAB and bifidobacteria.

| **Bacterial strains** | **Cholesterol reduction (µg/ml)** | |
|---|---|---|
| | **MRS broth** | **MRS broth+ 0.3% oxgall** |
| *L. paracasei* I321 | $14.98 \pm 3.34^{E,a}$ | $14.39 \pm 3.15^{N,a}$ |
| *L. rhamnosus* A1 | $22.29 \pm 5.42^{AB,b}$ | $34.22 \pm 1.99^{KL,a}$ |
| *L. rhamnosus* A2 | $25.36 \pm 6.09^{AB,a}$ | $30.30 \pm 3.83^{LM,a}$ |
| *L. rhamnosus* A9 | $9.73 \pm 6.66^{H,b}$ | $23.60 \pm 1.73^{M,a}$ |
| *L. rhamnosus* A10 | $17.04 \pm 4.48^{CD,b}$ | $43.15 \pm 3.45^{GH,a}$ |
| *L. casei* A27 | $21.82 \pm 2.70^{AB,B}$ | $42.58 \pm 1.67^{GH,a}$ |
| *L. casei* AN33 | $22.35 \pm 5.91^{AB,b}$ | $47.65 \pm 1.91^{EF,a}$ |
| *L. plantarum* 108 | $19.78 \pm 2.87^{BC,b}$ | $65.57 \pm 5.05^{A,a}$ |
| *L. plantarum* 178 | $6.85 \pm 3.49^{IJ,b}$ | $51.44 \pm 3.83^{CD,a}$ |
| *L. plantarum* A121 | $22.71 \pm 6.36^{AB,b}$ | $53.33 \pm 2.86^{BC,a}$ |
| *L. plantarum* A123 | $7.11 \pm 1.05^{IJ,b}$ | $44.27 \pm 3.22^{FG,a}$ |
| *L. plantarum* 124 | $18.66 \pm 4.78^{BC,a}$ | $28.24 \pm 5.14^{LM,a}$ |
| *L. plantarum* I61 | $12.26 \pm 5.86^{G,b}$ | $37.81 \pm 1.36^{JK,a}$ |
| *L. plantarum* 184 | $26.69 \pm 2.39^{A,b}$ | $54.14 \pm 1.69^{BC,a}$ |
| *Ent. faecalis* M118 | $12.26 \pm 6.43^{G,b}$ | $42.14 \pm 1.25^{GH,a}$ |
| *Ent. faecalis* M134 | $8.09 \pm 4.35^{IJ,b}$ | $55.90 \pm 4.34^{BC,a}$ |
| Ent. *faecalis* M237 | $21.65 \pm 5.95^{AB,b}$ | $40.85 \pm 5.23^{IJ,a}$ |
| *Ent. faecalis* M125 | $21.30 \pm 6.37^{AB,b}$ | $48.17 \pm 5.45^{EF,a}$ |
| *Ent. faecalis* M114 | $24.89 \pm 7.08^{AB,b}$ | $54.62 \pm 3.07^{BC,a}$ |
| *Ent. faecalis* M135 | $21.15 \pm 2.42^{AB,b}$ | $43.78 \pm 1.50^{GH,a}$ |
| *Ent. faecalis* M117 | $27.20 \pm 1.13^{A,b}$ | $51.98 \pm 4.81^{CD,a}$ |
| *Ent. faecalis* M145 | $21.36 \pm 4.49^{AB,b}$ | $49.28 \pm 3.79^{DE,a}$ |
| *Ent. faecalis* M126 | $24.44 \pm 1.96^{AB,b}$ | $53.65 \pm 4.37^{BC,a}$ |
| *Ent. faecium* EMA32.1 | $24.08 \pm 2.61^{AB,b}$ | $56.62 \pm 2.46^{BC,a}$ |
| *Ent. faecalis* EM17.3 | $14.35 \pm 5.42^{EF,b}$ | $57.54 \pm 4.31^{BC,a}$ |
| *Ent. faecium* EMA47.2 | $6.48 \pm 1.59^{IJ,b}$ | $58.96 \pm 3.83^{AB,a}$ |
| *Ent. faecium* EMA49.5 | $21.87 \pm 5.91^{AB,b}$ | $58.87 \pm 3.10^{AB,a}$ |
| *Ent. faecium* EMA31.6 | $7.23 \pm 1.39^{IJ,b}$ | $58.37 \pm 1.41^{AB,a}$ |
| *Ent. faecium* EMA410.4 | $14.98 \pm 1.47^{E,a}$ | $61.11 \pm 2.02^{AB,a}$ |
| *Ent. faecium* EMA31.8 | $9.35 \pm 4.26^{H,b}$ | $55.73 \pm 3.43^{BC,a}$ |
| *B. longum* AN37 | $14.15 \pm 0.51^{EF,b}$ | $52.19 \pm 0.86^{CD,a}$ |
| *B. longum* AN38 | $9.50 \pm 1.87^{H,b}$ | $52.47 \pm 1.83^{CD,a}$ |
| *B. bifidum* AN158 | $5.69 \pm 2.10^{J,b}$ | $44.24 \pm 1.39^{FG,a}$ |
| *B. bifidum* AN161 | $17.08 \pm 1.27^{CD,b}$ | $51.53 \pm 0.30^{CD,a}$ |

All data mean values of triple determination ± standard deviation (SD). Different superscript uppercase letters (A-N) indicate significant difference ($P < 0.05$) with the same column. Different superscript lowercase (a-b) letters indicate significant difference ($P < 0.05$) within the same row.

Table 19. Removal of cholesterol by resting and dead cells of LAB and bifidobacteria.

| Strains | Cholesterol reduction (mg/g) | |
|---|---|---|
| | **Resting cells** | **Dead cells** |
| *L. paracasei* I321 | $7.62 \pm 2.40^{H,a}$ | $4.81 \pm 0.55^{GH,a}$ |
| *L. rhamnosus* A1 | $10.28 \pm 0.76^{DE,a}$ | $6.04 \pm 1.67^{FG,b}$ |
| *L. rhamnosus* A2 | $10.53 \pm 0.56^{DE,a}$ | $5.69 \pm 1.05^{GH,b}$ |
| *L. rhamnosus* A9 | $10.05 \pm 0.64^{DE,a}$ | $6.87 \pm 1.10^{EF,b}$ |
| *L. rhamnosus* A10 | $11.39 \pm 1.47^{DE,a}$ | $4.97 \pm 0.75^{GH,b}$ |
| *L. casei* A27 | $10.53 \pm 1.16^{DE,a}$ | $3.87 \pm 0.71^{JK,b}$ |
| *L. casei* AN33 | $7.80 \pm 1.45^{H,a}$ | $6.26 \pm 0.71^{EF,a}$ |
| *L. plantarum* 108 | $10.31 \pm 1.62^{DE,a}$ | $6.04 \pm 0.30^{FG,b}$ |
| *L. plantarum* 178 | $14.62 \pm 1.77^{AB,a}$ | $5.04 \pm 0.70^{GH,b}$ |
| *L. plantarum* A121 | $12.46 \pm 1.59^{CD,a}$ | $4.79 \pm 0.47^{GH,b}$ |
| *L. plantarum* A123 | $11.12 \pm 0.75^{DE,a}$ | $4.31 \pm 1.44^{HI,b}$ |
| *L. plantarum* 124 | $8.47 \pm 3.86^{GH,a}$ | $6.95 \pm 1.85^{EF,a}$ |
| *L. plantarum* I61 | $14.82 \pm 4.77^{AB,a}$ | $3.48 \pm 0.62^{J,b}$ |
| *L. plantarum* 184 | $11.89 \pm 1.22^{DE,a}$ | $5.47 \pm 0.67^{GH,b}$ |
| *Ent. faecalis* M118 | $17.36 \pm 1.57^{AB,a}$ | $12.06 \pm 1.25^{AB,b}$ |
| *Ent. faecalis* M134 | $13.66 \pm 2.70^{BC,a}$ | $13.65 \pm 1.70^{A,a}$ |
| *Ent. faecalis* M237 | $12.02 \pm 1.77^{CD,a}$ | $8.18 \pm 1.15^{C,b}$ |
| *Ent. faecalis* M125 | $17.13 \pm 3.33^{AB,a}$ | $9.92 \pm 2.59^{BC,a}$ |
| *Ent. faecalis* M114 | $12.24 \pm 3.22^{CD,a}$ | $9.72 \pm 1.19^{BC,a}$ |
| *Ent. faecalis* M135 | $15.05 \pm 0.68^{AB,a}$ | $12.77 \pm 1.36^{AB,a}$ |
| *Ent. faecalis* M117 | $15.12 \pm 2.97^{AB,a}$ | $7.87 \pm 0.84^{CD,b}$ |
| *Ent. faecalis* M145 | $19.49 \pm 2.56^{A,a}$ | $11.83 \pm 2.21^{AB,b}$ |
| *Ent. faecalis* M126 | $11.01 \pm 2.19^{DE,a}$ | $6.90 \pm 1.30^{EF,b}$ |
| *Ent. faecium* EMA32.1 | $15.16 \pm 1.41^{AB,a}$ | $7.38 \pm 1.09^{DE,b}$ |
| *Ent. faecalis* EM17.3 | $14.31 \pm 3.18^{BC,a}$ | $7.77 \pm 1.90^{CD,b}$ |
| *Ent. faecium* EMA47.2 | $13.29 \pm 2.14^{BC,a}$ | $3.49 \pm 0.38^{J,b}$ |
| *Ent.faecium* EMA49.5 | $11.84 \pm 2.45^{DE,a}$ | $7.30 \pm 1.55^{DE,a}$ |
| *Ent.faecium* EMA31.6 | $8.35 \pm 1.05^{GH,a}$ | $4.90 \pm 1.98^{G,a}$ |
| *Ent.faecium* EMA410.4 | $13.91 \pm 1.42^{BC,a}$ | $10.81 \pm 1.68^{ABC,a}$ |
| *Ent. faecium* EMA31.8 | $10.04 \pm 1.42^{DE,a}$ | $7.82 \pm 1.05^{CD,a}$ |
| *B. longum* AN37 | $9.20 \pm 0.85^{FG,a}$ | $7.70 \pm 0.73^{CD,a}$ |
| *B. longum* AN38 | $10.51 \pm 3.31^{DE,a}$ | $6.31 \pm 1.36^{EF,a}$ |
| *B. bifidum* AN158 | $9.63 \pm 0.76^{EF,a}$ | $5.82 \pm 2.74^{GH,a}$ |
| *B. bifidum* AN161 | $11.80 \pm 0.68^{DE,a}$ | $3.53 \pm 0.13^{J,b}$ |

All data mean values of triple determination ± standard deviation (SD). Different superscript uppercase letters (A-J) indicate significant difference (P<0.05) with the same column. Different superscript lowercase (a-b) letters indicate significant difference ($P < 0.05$) within the same row.

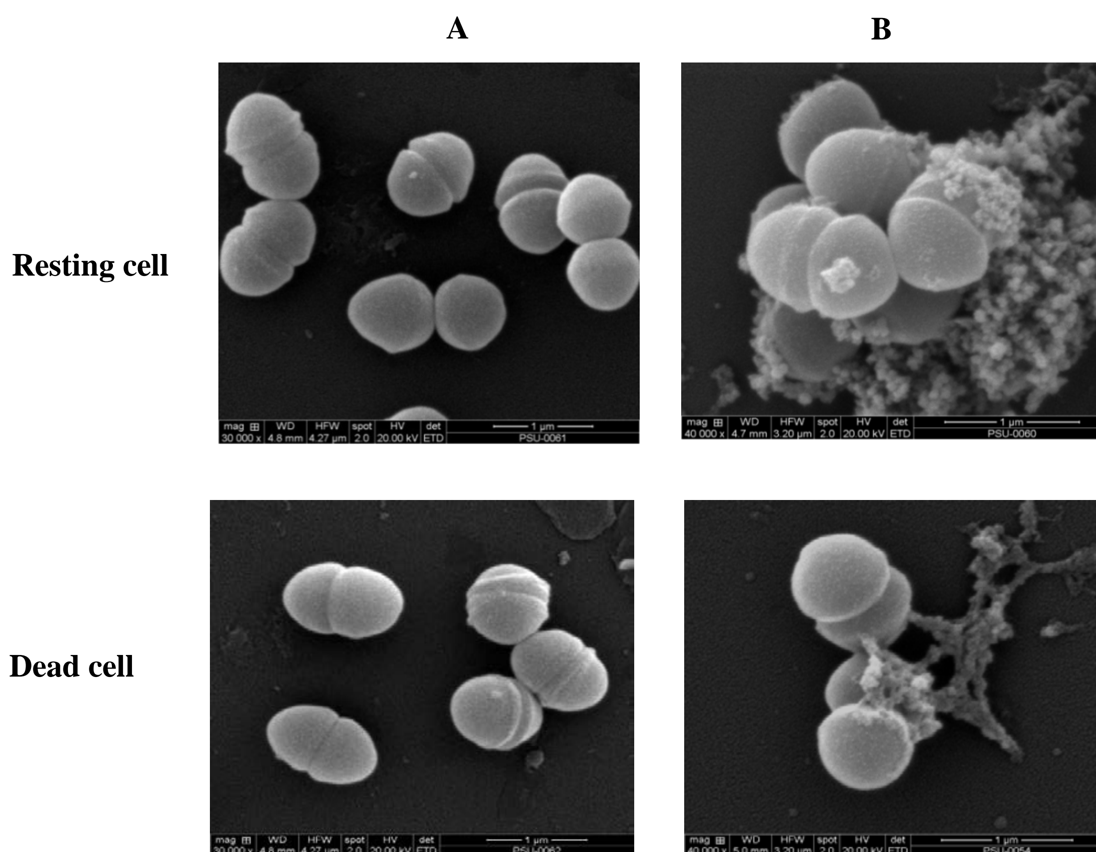


Figure 11. Scanning electron micrographs of resting and dead cell of *Enterococcus faecalis* M145 grown in the absence of cholesterol (A) and the presence of cholesterol (B) incubated at 37 ℃ for 24 h.

### 4.4.4 *In vitro* adhesion to the Caco-2 and HT-29 cell lines

Adhesion was tested using two epithelial cell lines of intestinal origin (Caco-2 and HT-29). The adhesion abilities of nine strains of LAB to epithelial intestinal cells Caco-2 and HT-29 are shown in Table 20. *Ent. faecium* EMA410.4 exhibited distinctly adhesion capacity to Caco-2 at higher level than the adherent epithelial strain (*B. bifidum* S17). *Ent. faecalis* M134 and *Ent. faecalis* EM17.3 were the most adherent strains to HT-29. Moreover, adhesion of LAB to HT-29 cell line was higher than Caco-2 cell line. These results showed that the studied strain reveals *in vitro* adherence ability to Caco-2 and HT-29 cell line. According to this study, *Ent. faecium* EMA410.4, *Ent. faecalis* M134 and

*Ent. faecalis* EM17.3 had greatly ability to produce BSH enzyme, remove cholesterol from the media during growth, remove cholesterol via binding to cellular surface of resting and dead cell and strongly adhered to Caco-2 and HT-29 cell lines. Therefore, these strains can be used in the future as a good candidate for lowering cholesterol level *in vivo*.

Table 20. Adhesion percentages of LAB to Caco-2 and HT-29 cell lines. *B. bifidum* S17 is positive controls of Caco-2 and HT-29 cell lines.

| Strains | Adhesion (%) | |
|---|---|---|
| | Caco-2 | HT-29 |
| *L. plantarum* 108 | $65.07 \pm 2.46^{D,b}$ | $71.36 \pm 0.31^{AB,a}$ |
| *Ent. faecalis* M134 | $66.44 \pm 1.66^{CD,b}$ | $74.74 \pm 2.32^{A,a}$ |
| *Ent. faecium* EMA32.1 | $70.95 \pm 0.63^{BC,a}$ | $72.01 \pm 0.49^{AB,a}$ |
| *Ent. faecalis* EM17.3 | $70.13 \pm 2.11^{BC,b}$ | $74.84 \pm 0.72^{A,a}$ |
| *Ent. faecium* EMA47.2 | $72.32 \pm 0.70^{AB,a}$ | $72.58 \pm 0.53^{AB,a}$ |
| *Ent. faecium* EMA49.5 | $68.67 \pm 1.42^{BC,a}$ | $69.93 \pm 0.32^{AB,a}$ |
| *Ent. faecium* EMA31.6 | $70.26 \pm 1.31^{BC,a}$ | $72.48 \pm 0.37^{AB,b}$ |
| *Ent. faecium* EMA410.4 | $75.35 \pm 1.47^{A, a}$ | $71.12 \pm 1.23^{AB,b}$ |
| *Ent. faecium* EMA31.8 | $68.31 \pm 3.27^{BC,a}$ | $69.90 \pm 1.06^{AB,a}$ |
| *B. bifidum* S17 | $73.01 \pm 1.14^{AB,a}$ | $72.26 \pm 0.85^{AB,a}$ |

All data mean values of triple determination ± standard deviation (SD). Different superscript uppercase letters (A-D) indicate significant difference ($P < 0.05$) with the same column. Different superscript lowercase (a-b) letters indicate significant difference ($P < 0.05$) within the same row.

**4.5 Discussions**

There is growing interest in the preventing and lowering hypercholesterolemia by probiotic such as *Lactobacillus*, *Enterococcus* and *Bifidobacterium* has emerged recently (Lye *et al*., 2010; Kumar *et al*., 2012; Helim *et al*., 2016). This study demonstrated that nine strains of *Ent. faecalis* and five strains of *Ent. faecium* were able to excrete BSH enzyme to deconjugate sodium salt of taurodeoxycholic acid (TDCA). BSH enzyme is able to hydrolyze conjugated taurodeoxycholic acid leading to amino acid residues and deconjugated bile acid resulting to deconjugated bile acid precipitating at low pH by showing precipitation zone on MRS agar supplemented with 0.5% (w/v) TDCA. Cholesterol is the precursor of primary bile salts that are synthesized in the liver and are stored and concentrated as conjugated bile salts in the gall bladder (Liong and Shah, 2005; Begley *et al*., 2006). BSH enzyme catalyzes the hydrolysis of glycine-and taurine-conjugated bile salts into amino acid residues and unconjugated or deconjugated bile acids in the enterohepatic circulation (Moser and Savage, 2001; Wijaya *et al*., 2002; Liong and Shah, 2005; Parvez *et al*., 2006). The deconjugated bile salts are less absorbable and therefore can be excreted in the feces at large amounts in the form of free bile acids. Thus, leads to a reduction of serum cholesterol level either by *de novo* bile acid synthesis from cholesterol in an attempt to compensate bile acid loss, or by reduced cholesterol solubility and absorption through the intestinal lumen. Therefore, more cholesterol is required to synthesize new bile acids (Kumar *et al*., 2012; ref). Several studies demonstrated that *Ent. faecalis* and *Ent. faecium* were able to excrete BSH enzyme (Franz *et al*., 2001; Knarreborg *et al*., 2002 Wijaya *et al*., 2004; Guo *et al*., 2016). Moreover, the presence of BSH enzyme in probiotics renders them more tolerant to bile salt resulting to their survival in GI tract, which also helps to reduce the cholesterol level in the host (Moser and Savage, 2001; Patel *et al*., 2010).

The cholesterol assimilation or up-take by growing cells could reduce the amount of cholesterol available for absorption from the intestine thus making it unavailable for absorption from the intestines into the blood (Walker and Gilliland, 1993; Pigeon *et al*., 2002; Liong and Shah, 2005). Several strains of *Lactobacillus*, *Bifidobacterium* and

*Enterococcus* were able to remove cholesterol from the medium during growth (Pereira and Gibson, 2002; Sirilun *et al*., 2010; Guo *et al*., 2012). According to this study, the levels of cholesterol assimilation of LAB and bifidobacteria in the medium containing 0.3% bile salt were higher than the medium without bile salt. *L. plantarum* 108, *E. faecium* EMA410.4, *E. faecium* EMA47.2, *E. faecium* EMA49.5 and *E. faecium* EMA31.6 were significantly higher cholesterol removal ability in the MRS broth medium supplemented with 0.3% bile salt than other strains. Bile salt is biological surfactants that could lower the surface and interfacial tension, leading to increased cellular attachment of cholesterol. The emulsifying feature of bile affected cholesterol removal. The level of cholesterol reduction of LAB and bifidobacteria in the medium supplemented with each bile concentration (1-3 mg/ml) was higher than in the medium without bile (Tok and Aslim, 2010). In addition, cholesterol assimilation was associated with the presence of bile salts and cholesterol removal from the medium increased with increasing concentration of bile salt (Lye *et al*., 2010). This result is in agreement with several studies reported that LAB and bifidobacteria are able to remove cholesterol from cultured medium during growth in the presence of bile acids (Tahri *et al*., 1997; Pereira *et al*., 2002; Lim *et al*., 2004, Lye *et al*., 2010). According to this study, resting cells and dead cells of all LAB and bifidobacteria strains were able to remove cholesterol in MRS broth supplemented with 0.3% (w/v) oxgall. Cholesterol removal capacity of resting and dead cells implied that cholesterol may be removed via binding onto the bacterial cellular surface (Kimoto *et al*., 2002; Zeng *et al*., 2009; Lye *et al*., 2010; Tok and Aslim, 2010; Miremadi *et al*., 2014; Choi *et al*., 2015). Binding of cholesterol to the cell surface may be a physical phenomenon and be related to the cell membrane, bile exposure in bacteria leading to changes in the lipid composition of bacterial membranes (Zavaglia *et al*., 2002; Taranto *et al*., 2003). Cholesterol might form hydrogen bonding with the amide N-H group of the bile acids and the oxygen molecules of sugar hydroxyl group in the membrane lipid, forming an intermolecular bonding network (Boggs, 1987). Moreover, resting cells exhibited higher cholesterol removal compared with dead cells. The binding of cholesterol onto bacterial cell surface was observed using SEM. The bounded

cholesterol particle on the cell surface resulted in the roughness of cell wall. In addition, large amounts of cholesterol adhered on the cell surface of resting cell. On the contrary, small amounts of cholesterol adhered on the cell surface of dead cell. This result correlated with cholesterol removal ability of resting cells was higher than dead cells. Likewise several studies demonstrated that resting cells exhibited higher cholesterol removal compared with dead cells (Lye *et al*., 2010; Choi *et al*., 2015).

Bacterial adhesion to the intestinal mucosa and human epithelial cells influences the residence time (O'Sullivan *et al*., 1992; Munoz-Provencio *et al*., 2009) and promoting probiotic cholesterol lowering affect *in vivo* (Tomaro-Duchesneau *et al*., 2015). This study revealed that *Ent. faecium* EMA410.4, *Ent. faecalis* M134 and *Ent. faecalis* EM17.3 strongly adhered to Caco-2 and HT-29. Epithelial cells, Caco-2 and HT-29 cell lines were isolated from human colonic adenocarcinoma have been most widely used models to assess the adhesion ability of probiotic *in vitro* as they closely mimic the normal intestinal epithelium (Gagnon *et al*., 2013; Sharma and Kanwar, 2017). Several studies have carried out using Caco-2 and HT-29 to screen the adherence properties of probiotic strains (Matijasic *et al*., 2003; Laparra and Sanz, 2009; Martin *et al*., 2012). In addition, adhesion of LAB to HT-29 cell line was higher than Caco-2 cell line. The presence of mucus may play an important role in bacterial adhesion (Tassell and Miller, 2011). HT-29 cell line contains a small proportion of mucus-secreting cells, while Caco-2 cell line does not contain mucus-secreting cells (Sharma and Kanwar, 2017).The epithelial cells of the gastrointestinal tract are covered by a layer of mucus which is the first physical barrier to the host-cell stimulation by bacteria in the gut (Tassell and Miller, 2011; Cornick *et al*., 2015). This result was agreed with several studies that bacterial adhesion to HT-29 was higher than Caco-2 cell lines (Gagnon *et al*., 2013; Sharma and Kanwar, 2017).

**4. 6. Conclusion**

Thirty-four strains of LAB and bifidobacteria were able to reduce cholesterol in the medium containing 0.3% bile salt was higher than the medium without bile salt. *L. plantarum* 108 exhibited the highest cholesterol reduction. In addition, The BSH activity was only confined among nine strains of *Ent. faecalis* and five strains of *Ent. faecium*. The cholesterol binding ability on the cell surface of the resting cells and dead cells was exhibited with all LAB and bifidobacteria. The strain of *Ent. faecalis* M145 achieved the highest cholesterol removal by resting cells and *Ent. faecalis* M134 exhibited the highest cholesterol removal by dead cell. All strains exhibited higher cholesterol removal when cells were resting compared with and dead cells. Furthermore, *Ent. faecium* EMA410.4, *Ent. faecalis* M134 and *Ent. faecalis* EM17.3 strongly adhered to Caco-2 and HT-29 cell line.

# CHAPTER 5

## SUMMARY

Among 6 healthy newborn infants, infant age and feeding type were the most influential factor for the population of bifidobacteria, clostridia, bacteroides and eubacteria, whereas feeding type and delivery mode were the main factors influencing total bacteria population. Moreover, the exclusive breast-feeding greatly supported the bifidobacterial growth. Formula milk seemed to enhance the growth of clostridia and bacteroides. The highest level of the clostridia was found in Cesarean born, exclusively formula fed infant (FF1-F). In addition, the amount of bacteroides was increased in Cesarean born, formula fed infant (FF2-H) at the first week to 5 months of age. PCR-DGGE analysis demonstrated that there was change in the bifidobacterial community composition associated with feeding type and delivery mode. The high numbers of bifidobacteria band were observed in both naturally born, exclusively breast fed infant (BF1-D) and naturally born, combination of breast and formula fed infants (CF1-A). The highest number of bifidobacterial species was observed in both naturally born, exclusively formula fed infant (FF1-F) and naturally born, combination of breast and formula fed infants (CF1-A). The most common bifidobacterial species found in healthy infants were *Bifidobacterium* sp. and *B. longum.*

The number of fecal LAB from the infants fed with the combination diet of breast milk and a formula containing FOS-GOS (CF1-A infant) and inulin-GOS (CF2-C infant) was significantly higher than that from both exclusively breast-fed (BF1-D infant and BF2-E infant) and exclusively formula fed ones (FF1-F infant and FF2-H infant). Moreover, the band richness of LAB population in infants with combination (CF1-A) infant and CF2-C infant) and exclusive formula feeding (FF1-F infant and FF2-H infant) and the exclusively breast-fed infants (BF2-E) was not different. Type of feeding had not influence on LAB diversity. The most number of LAB species was found in the infant with the exclusive breast fed infant (BF2-E infant) and exclusive formula fed diet

supplemented with FOS-GOS (FF1-F infant). Moreover, uncultured bacteria were commonly found in infants. Combination feeding was able to support the growth of *L. plantarum* and *Leu. mesenteroides.* The exclusive feeding with formula supplemented with FOS-GOS enhance growth of *L. rhamnosus* and *L. gasseri*, while the formula supplemented with inulin-GOS sustained growth of *L. gasseri*, *L. fermentum* and *L. paracasei.* In addition, exclusive breast milk feeding was capable to promote the growth of *L. gasseri*, *L. crispatus*, *L. plantarum* and *L. helveticus*.

Thirty-for strains of LAB and bifidobacteria were able to assimilate cholesterol in the 0.3 % oxgall bile containing medium in the range of 14.39-65.57 µg/ml using *O*-phthalaldehyde method. *L. plantarum* 108 exhibited the significant highest assimilation. The bile salt hydrolase (BSH) activity was only confined among nine strains of *Ent. faecalis* and five strains of *Ent. faecium*. The cholesterol binding ability was tested with all LAB and bifidobacteria of resting cells and dead cells range of 7.62-19.49 mg/g dry weight and 3.48-13.65 mg/g dry. Furthermore, *Ent. faecium* EMA410.4, *Ent. faecalis* M134 and *Ent. faecalis* EM17.3 strongly adhered to Caco-2 and HT-29 cell line.

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**List of Publication and Proceeding**

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