MATERNAL NUTRITION AND ITS INFLUENCE AFFECTING THE DEVELOPMENT OF INFANT GUT MICROBIOTA IN HONG KONG

YEUNG OI YEE

PhD

The Hong Kong Polytechnic University

2023

The Hong Kong Polytechnic University
Department of Applied Biology and Chemical Technology

Maternal Nutrition and Its Influence Affecting the Development of Infant Gut Microbiota in Hong Kong

Yeung Oi Yee

A thesis submitted in partial fulfillment of the requirements for
the degree of Doctor of Philosophy
May 2023

CERTIFICATE OF ORIGINALITY

I hereby declare that this thesis is my own work and that, to the best of my knowledge and belief, it reproduces no material previously published or written, nor material that has been accepted for the award of any other degree or diploma, except where due acknowledgment has been made in the text.

Signature: _____

Name of Student: Yeung Oi Yee

Abstract

The infant gut microbiome plays a vital role in health. The disruption of early colonization may increase the risk of diseases and inflammatory immune response. Maternal nutrition and early infant feeding mode affect infant gut microbiota development. Breast milk and its alternatives are generally the only food for infants in the first 4 – 6 months. Breast milk contains human milk oligosaccharides (HMO) and milk microbiota, which may bring health benefits to infants. Research studies showed that maternal nutrition may also affect the HMO content in breast milk.

There were studies on the influence of maternal diet during pregnancy on infant gut microbiota, yet the effects of maternal diet during lactation were scarce. And the information related to gut microbiota development and its association with maternal nutrition and HMO in the breast milk of Hong Kong infants was limited. The objectives of this study are: Identify the characteristic of the maternal diet during pregnancy and lactation in Hong Kong; Examine the development of the gut microbiota of infants in Hong Kong during the first year of life; Investigate the effects of maternal diet and early feeding practice on infant gut microbiota and assess the HMO concentration in breast milk and its correlation between maternal diet and infant gut microbiota.

In this research study, pregnant and lactation women were recruited for diet assessment by Food Frequency Questionnaire and 3-day diet record, respectively, to assess the maternal nutritional status during pregnancy and lactation in Hong Kong. Infant fecal and breast milk samples were collected to study the gut microbiota development in Hong Kong infants, HMO in breast milk, and if maternal nutrition influences the HMO concentration in breast milk and infant gut microbiota development.

The diet analysis showed that the average intake of vegetables, fruits, and dairy products in pregnant and lactating women was insufficient. One of the major problems is inadequate dietary fiber intake. The majority of the pregnant and lactating women had less than 50% of the recommended dietary fiber intake. Since most mothers took nutritional supplements during lactation, most micronutrient requirements except Vitamin A and Calcium could be met.

16S rDNA sequencing was conducted to analyze the gut microbiota development in infants in Hong Kong during the first year. Alpha diversity of infant gut microbiota was lower at two months of age than those at older ages. The phyla Bacteroidetes, Firmicutes, Proteobacteria, and Actinobacteria were the dominant bacteria across the first year. In addition, a higher abundance of *Bifidobacterium* in the fecal samples were detected in the group of exclusively breastfed infants at month 2 and 4. However, this observation disappeared from the 6th month and this result could be due to the changes in the gut microbiota initiated by the introduction of solid food, which gradually reduced the profound effect of breastfeeding.

Correlational analyses on maternal diet during lactation and infant gut microbiota were conducted. Total fiber intake was positively associated with the Family Tannerellaceae. Intake of medium-chain saturated some fatty acids was positively correlated genera *Escherichia-shigella*. There was also a significant positive association between polyunsaturated fatty acid intake and *Klebsiella*. *Escherichia and Klebsiella* are linked with chronic low-grade inflammation. A maternal diet with lower fat content may be beneficial to infants.

Our results showed that the HMO concentration was dynamic throughout the lactation period, and the most abundant HMO was 2′-fucosyllactose (2′FL). Lacto-N-neotetraose (LNnT) was the highest HMO concentration in breast milk at month 2 and reduced afterward. Total HMO concentration reduced throughout the lactation period. In our study, dietary fiber intake, including soluble and insoluble dietary fiber, was found to have a positive correlation with LNnT, while fruit intake was associated positively with 2'FL in human milk. The result implied that high fiber or fruit intake might associate with a high level of LNnT and 2′FL in breast milk. However, the fruit and dietary fiber intake in Hong Kong during lactation was low.

To conclude, infant gut microbiota development is variable and shaped by different factors, including maternal diet and early feeding mode. The findings of this study could provide scientific evidence to draw the attention of public health to pay attention to adopt a healthy diet during pregnancy and lactation, which promotes good quality breast milk that benefits the development of healthy gut microbiota in Hong Kong infants.

Acknowledgments

I would like to express my sincere gratitude to my supervisors, Prof Man-Sau Wong and Dr. Amber Chiou, for providing guidance and advice throughout the study period, which lasted for more than six years.

I am incredibly thankful to Ms. Erica Ho, Ms. Fanny Ng, and Ms. Vincy Wong for coordinating the subject recruitment and sample collection. In addition, Ms. Liwen, Ms. Song Ang-Xin, and other laboratory members for helping me with the laboratory work, including HMO analysis and DNA extraction from samples.

Special thanks are also given to Mr. Theo Lam and Dr. Perry Shum for the data extraction of 16S rRNA sequencing. I also wish to thank Dr. Kenneth Lo for the data and statistical analysis advice. I am also thankful to Prof Zhu Hui-lian and Dr Sunny Wong for providing valuable comments and feedback on my research which help improving the quality and clarity of my project.

In addition, I would like to express my gratitude to Prof Tam Wing Hung and his team at Prince of Wales Hospital and breastfeeding-supporting organizations for helping recruit subjects at Prince of Wales Hospital and online ,repectively.

And finally, I would like to express my deep appreciation to all my family members for their support.

Table of Contents

List of Figures

Chapter 2

Chapter 4

Chapter 5

Chapter 6

List of Tables

Chapter 4

Chapter 6

Abbreviations

2'FL	2'-Fucosyllactose
3FL	3-Fucosyllactose
FSL	3-fucosyl-3'-sialyllactose
3'SL	3'-sialyllactose
6'SL	6'-sialyllactose
AMDR	Acceptable Macronutrient Distribution Range
ACN	Acetonitrile
BMI	Body mass index
CDC	Centers for Disease Control and Prevention
CHP	Centre of Health Protection
CNS	Chinses Nutrition Society
DH	Department of Health
DRI	Dietary Reference Intake
DFLNT	Difucosyllacto-N-tetrose
DFL	Difucosyllactose
DSLNT	Disialyllacto-N-tetraose (
FFQ	Food Frequency Questionnaire
FOS	Fructooligosaccharide
FLNH	Fucosyllacto-N-hexaose
FUT 2	Fucosyltransferase 2
FUT 3	Fucosyltransferase 3
GOS	Galactooligosaccharide
HMO	Human milk oligosaccharide
HILIC	Hydrophilic interaction liquid chromatography
IOM	Institute of Medicine
LNDFH I	Lacto-N-difucohexaose I
LNFP I	Lacto-N-fucopentaose I
LNFP III	Lacto-N-fucopentaose III
LNH	Lacto-N-hexaose
LNnH	Lacto-N-neohexaose
LNnT	Lacto-N-neotetraose
LNT	Lacto-N-tetraose
LNT II	Lacto-N-triose II
LPS	Lipopolysaccharide
MS	Mass spectrometry

MRM	Multiple reaction monitoring
OTUs	Operational Taxonomic Units
RDA	Recommended Dietary Allowance
RNI	Recommended Nutrient Intake
SFCA	Short chain fatty acid
LSTa	Sialyllacto-N-tetraose a
SPE	Solid phase extraction
SPSS	Statistical Package for Social Sciences
TLR4	Toll-like receptor 4
TFA	Trifluoroacetic acid
UPLC/QqQ-MS	Ultraperformance liquid chromatography triple quadrupole-mass spectroscopy
WHO	World Health Organization

Chapter 1 Introduction and Objectives

There are more than trillions of bacteria in our gut, which are suggested to have a significant role in metabolic functions and the immune system (Olin et al., 2018). Gut microbiota has been shown to link with human health. The first colonization of microorganisms in the gut occurs at birth and gradually matures and develops until three years old (Milani et al., 2017). The gut microbiota is relatively stable by age three and becomes adult-like (Rinninella et al., 2019). It has been reported that disruption of early gut microbiota colonization may increase the risk of asthma, allergy, and immune-inflammatory response (Gensollen et al., 2016). Therefore, gut microbiota in the infant affects gut health directly and future growth, such as the risk of obesity and other diseases (Butel et al., 2018). There are many factors affecting the development of infant gut microbiota, including the nutrition status of mothers during pregnancy (Garcia-Mantrana et al., 2020), lactation (Alsharairi, 2020), and early infant feeding practices during the postnatal period (Ho et al., 2018).

Inadequate vegetable and fruit consumption but relatively high fish and seafood consumption are some characteristics of the diet in Hong Kong. According to the Report of Health Behaviour Survey 2018/19 published by the Centre of Health Protection (CHP), 95.6% of people aged 15 or above did not have an adequate daily intake of fruit and vegetables when considering the World Health Organization (WHO) recommendation which the daily fruit and vegetable consumption should be at least five servings per day (CHP, 2020). According to the Report of the Second Hong Kong Population-Based Food Consumption Survey conducted by the Centre for Food Safety and the Department of Health, the average consumption of milk and dairy products was

24.86 g/day (DH, 2021). The consumption level was much lower than 1-2 serving a day.

However, seafood and fish are one of the popular choices in Hong Kong's diet. The consumption was 71.8kg per capita, ranking second in Asia (WWF, 2020). The consumption level was much higher than in the global which the global per capita fish consumption was estimated at 20.3 kg in 2017 (FAO, 2021). It is worth examining the characteristics of maternal diet during pregnancy and lactation in Hong Kong. Since there is not much data published in Hong Kong, one of the aims of this study is to assess the maternal diet during pregnancy and lactation.

Metagenomics analysis has been carried out on fecal samples of infants in the United State of America and Europe to understand the development of infant gut microbiota. However, there is not much data in Hong Kong. Therefore, one of the objectives of this study is to examine the development of gut microbiota during the first year of life and the factors affecting the development.

WHO recommends exclusively breastfeeding for the first six months, followed by solid food introduction with continued breastfeeding for up to 2 years old or after (WHO, 2017). Breast milk or infant formula is the first nutrient source for the infant, and it is one of the factors modifying the gut microbiota in infants (Serino et al., 2017). *Bifidobacterium* and *Lactobacillus* in the feces of Breast-fed infants were significantly higher than formula-fed infants, while the Bacteroidetes, Clostridium, and Enterobacter level was lower (Backhed et al., 2015). It is worth investigating the effects of feeding practice in early life on the gut microbiota of infants in Hong Kong.

It has been reported that intake of dietary fiber, fruit, vegetable, and fish consumption may affect the diversity and composition of the gut microbiota. Some studies investigated the effects of maternal diet during pregnancy on infant gut microbiota. (Babakobi et al., 2020; Garcia-Mantrana et al., 2020; Lundgren et al., 2018). Yet, the effects of maternal diet during lactation were limited (Sindi et al., 2021; Sindi et al., 2022). With the dietary information from mothers, a deeper understanding of the relationship between dietary habits during lactation and the gut microbiota in infants may be able to develop in this study.

There is a possibility that the maternal diet during lactation may influence the human milk oligosaccharide (HMO) content and the bacterial composition of human milk and pose downstream effects on the infant gut microbiota (Sindi et al., 2021). The proposed reasons were that breast milk contains HMO acting as prebiotics and the microbiome, which may directly transfer to the baby(Corona-Cervantes et al., 2020). Breast milk and lactation diet information are useful to investigate the HMO level in breast milk and if there was any association between maternal diet during lactation and HMO production. In order to find out if there are any downstream effects on the infant gut microbiota, the correlation between HMO present in breast milk and the infant gut microbiota is worth investigating.

Gut microbiota is a hot topic research field since it is highly associated with the risk of diseases and health prevention. Maternal nutrition and feeding pattern in infants 'early life are some factors that may affect infant gut microbiota. The characteristic of Hong Kong's diet is notable: fish and seafood consumption are high, but vegetable, fruit, and dairy products are low. However, not much data on mothers' diets during prenatal and

postnatal diets and infant microbiota in Hong Kong could be found. In the present study, by analyzing the microbiota in stool samples and HMO content in breast milk and collecting the demographic and dietary information of both mothers and infants, we may identify an association between the nutrient source and gut microbiota growth in infants. Therefore, if an association could be found, we may be able to draw out some strategies for modifying mothers' diet and feeding patterns in an infant's early life to favor the early gut microbiota establishment and lower the risk of diseases in the later life of the next generation. We hypothesized that maternal diet and early feeding practice during the postnatal period would affect the HMO concentration in breast milk and gut microbiota development in Hong Kong infants.

Objectives of the research study:

To

- Identify the characteristic of the maternal diet during pregnancy and lactation in Hong Kong.
- Examine the development of the gut microbiota of infants in Hong Kong during the first year of life.
- Investigate the effects of maternal diet and early feeding practice on infant gut microbiota.
- Assess the HMO concentration in breast milk and its correlation between maternal diet and infant gut microbiota.

Chapter 2 Literature Review

2.1 Developmental Programming

David Barker is one of the earliest scientist to propose a concept called "Developmental origins of health and diseases" (Barker, 2007). Stimuli during the perinatal period affect the development of the fetus/infant, which imposes a long-term effect on the health of their future life. The stimulus which will pose the influences on the development of the next generation during pregnancy is shown in Figure 2.1.

Maternal nutrition is one of the stimuli which may affect the risk of diseases in the future life of the next generation through epigenetic mechanisms. It was proposed that gestation undernutrition may induce compensation growth in early life and increase the risk of obesity and type 2 diabetes (Stein et al., 2019), while maternal obesity, dietary intake of mothers during pregnancy, and infant feeding practices will also alter the risk of obesity and type 2 diabetes in the offspring (Perng et al., 2019).

2.2 Developmental Programming and Development of Infant Gut Microbiota

From pregnancy to two years of age, roughly the first 1000 days of life, is a critical window for development and growth. Nutrient intake can shape the gut microbiome, which may induce epigenetic changes that alter disease programming (Lee, 2019). The Figure 2.2 shows the factors which may modulate the microbial composition and thus affect the disease status. The detail of each factor will be discussed in section 2.7 below. The basic information about human gut microbiota will be introduced first.

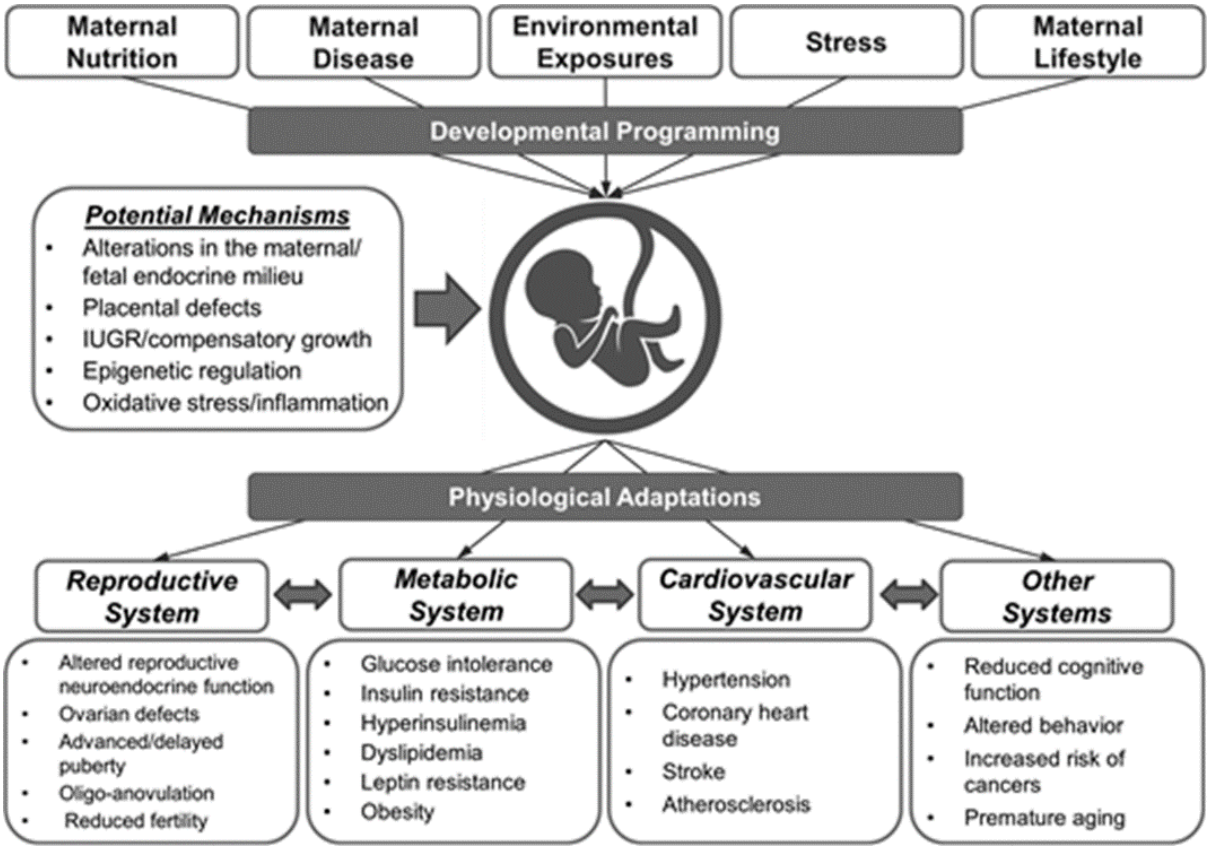


Figure 2. 1 Factors affecting the development of the next generation (Padmanabhan et al., 2016)

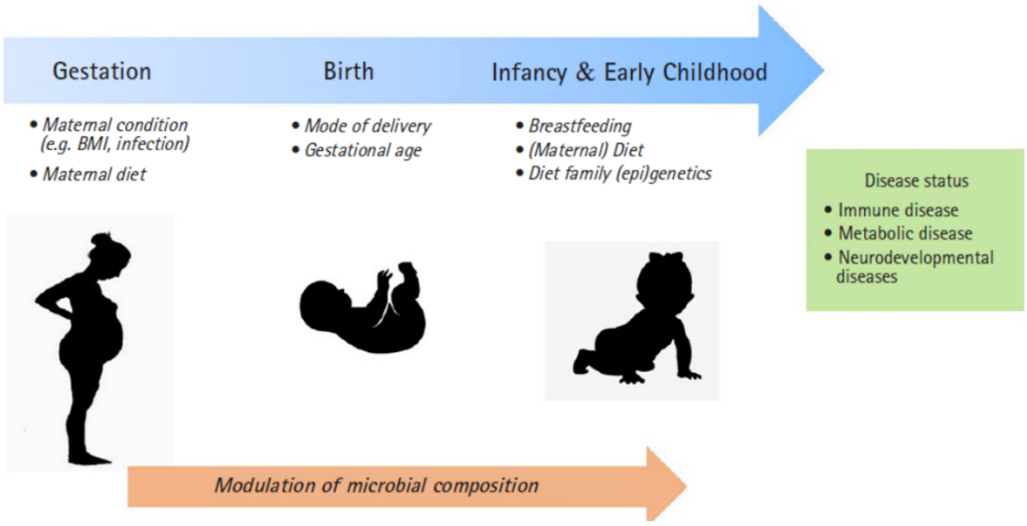


Figure 2. 2 Factors of modulation of microbial composition. (Lee, 2019)

2.3 Gut Microbiota in Human

Bacteria, yeast, and viruses are common species of microorganisms present in human gut, forming gut microbiota (Rinninella et al., 2019). More than trillions of microbiomes are in the human gastrointestinal tract, and they plays a role in metabolic functions and health (Olin et al., 2018). For example, digestion can be regulated by the bacteria in the gut, and beneficial bacteria in the gut may help improve immune system by inhibiting the growth of pathogenic bacteria (Rinninella et al., 2019). Bacteria are classified into phylum, classes, order, families, genera, and species (high to low). Figure 2.3 shows the taxonomic gut microbiota composition. 90% of gut microbiota comprises Firmicutes and Bacteroidetes which are the two major phyla.

2.4 Gut Microbiome in Infant and Its Origin

The four most prevalent phyla found in infants' gut are Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria (Rinninella et al., 2019). It is proposed that the origin of the microbiota in infants should be from the mother, which is acquired by vertical transmission (Yassour et al., 2018). Traditionally it is postulated that the developing fetus grows in an almost sterile environment which is isolated from most of the microorganisms in the womb of mothers (Perez-Munoz et al., 2017), the exposure to the first inoculum of microbes is during birth (Dalby & Hall, 2020). Milani et al. (2017) suggested that the first colonization of microorganisms in the gut occurs at birth and gradually matures and develops until three years old.

However, there is still no conclusion on the first inoculum of bacteria in infants, since a study found that there were bacteria in the fatal gut before birth. They proposed that

the acquisition may begin in utero (Perez-Munoz et al., 2017) from the oral and vaginal microbiome in mothers. The microbiome in mothers may translocate into the fetus during pregnancy. Figure 2.4 shows the traditional hypothesis on the origins of neonatal microbiota vs. the recent hypothesis.

2.5 Establishment of the Gut Microbiota in Infant

Infants acquire the first inoculum of microbes from their mothers. Then after birth, infants will be exposed to more different species of bacteria, replacing the initial more aerobic ones with anaerobic ones (Backhed et al., 2015). The development of gut microbiota in infants was proposed to divide into different phases, such as the first 1-2 weeks after birth, and the introduction of solid foods at 6 months of life, will also influence the composition of the infant gut microbiota (Bharadia et al., 2020). One of the earliest studies conducted by Stark and Lee (1982) found that the introduction of solid foods resulted in an increase in enterobacteria and enterococci. Then *Bacteroides spp.,: Clostridia,* and *Streptococci* started colonizing in the gut.

Gut microbiota in infants will keep developing, and the changes is shown in Figure 2.5. The alpha diversity increased when the infant grew, and the gut microbiota became more complex. When compared to adults' gut microbiota with the growing infant, there was less dissimilarity over time (Backhed et al., 2015). The composition of gut microbiota in the first year of life is indicated in Figure 2.6, the composition is more complex, and the abundance of each species keeps changing. In the first 1 -2 months, Bifidobacterceae was the dominant one; however, when it was the age of 3, the dominant one was Bacteriodaceae (Drago et al., 2019). Usually, it may take the first three years of life to develop an adult-like stable gut microbiota system; 60 – 70% of the system will remain stable throughout the whole life (Kashtanova et al., 2016).

Figure 2. 3 Examples of microbiota composition in the gut. (Rinninella et al., 2019)

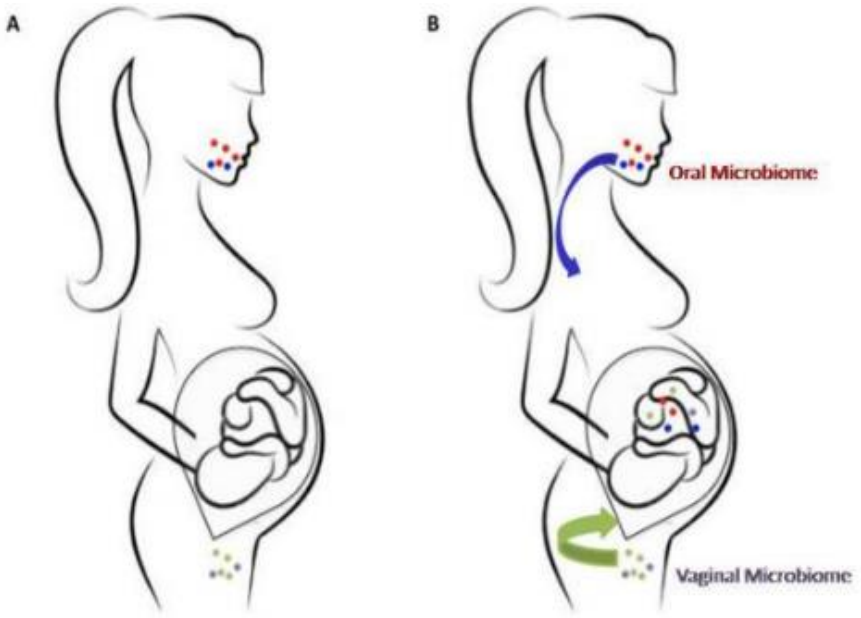


Figure 2. 4 Hypothesis of the origin of infant microbiota

(A) The uterus has been considered a sterile womb for years. (B) Recent studies have questioned this hypothesis: Microbial colonization may already begin in utero. (D'Argenio, 2018).

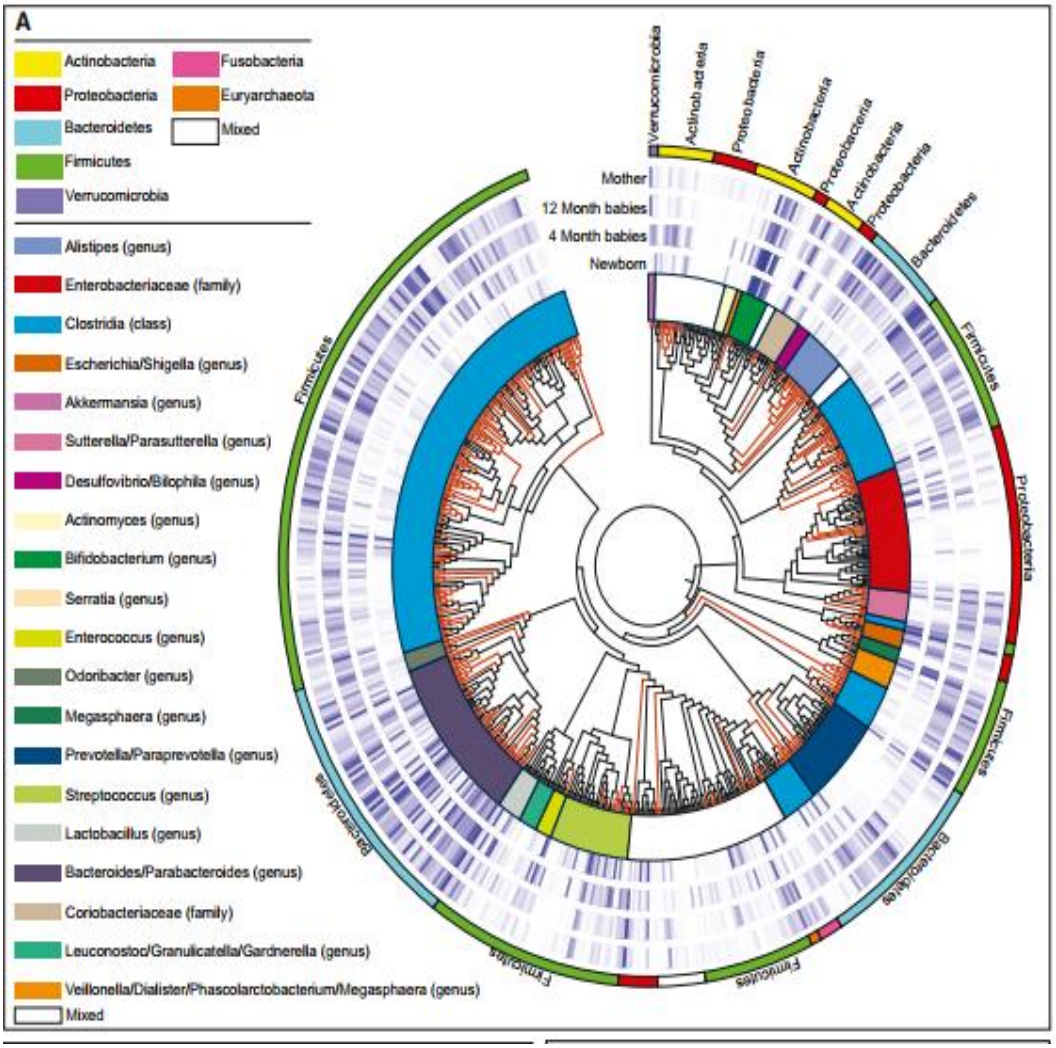


Figure 2. 5 Phylogenetic Tree of the MetaOTUs and Differences in the Fecal Microbial Communities of Newborns, 4-Month-Old and 12-Monthold, Infants, and Mothers (Backhed et al., 2015).

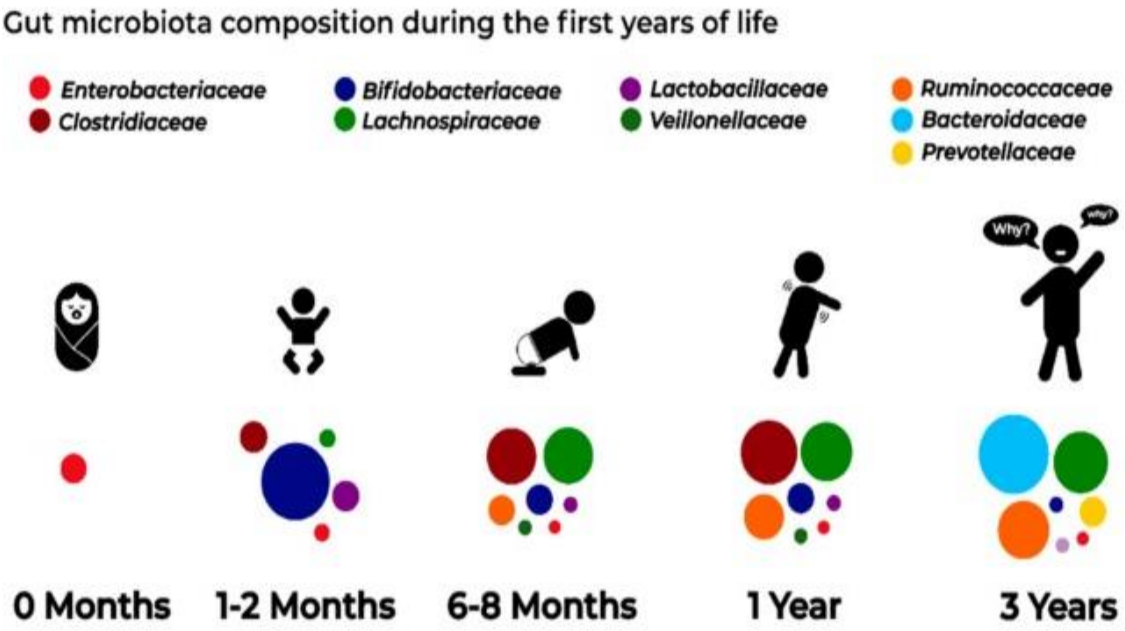


Figure 2. 6 Gut microbiota during the first year of life. (Drago et al., 2019).

2.6 Gut Microbiota and Health

Dysbiosis of gut microbiota in infants will not only affect gut health directly but also the future growth, (Butel et al., 2018). Dysbiosis of infant gut microbiota in early life increases the risk of diseases in future life, such as asthma, allergy, and immune-inflammatory response (Tamburini et al., 2016).

Obesity

A study was conducted to assess the stool of both lean and overweight/obese children. It was found that the abundance of *Prevotella* was lower and *Clostridium* was higher in obese children when compared with the children with normal weight (Barczynska et al., 2018) (Figure 2.7). While a study suggested that Proteobacteria play a role in obesity because they can produce pro-inflammatory lipopolysaccharides and enhance the host fat storage (Rizzatti et al., 2017). Since Proteobacteria are suggested to be associated with the risk of metabolic diseases in adults, they are important to the development of immune system in the infant. Early colonization of microbes in the gut and, subsequently, changing to a more adult-like stable gut microbiota is a normal process over time; however, if this process is disrupted, it may increase the risk of diseases in infants, especially the preterm group (Neu, 2016).

Diversity and richness of the gut microbiota are also associated with health; it is suggested that there is an association between body mass index (BMI) and obesity (Tomova et al., 2019). A reduction in microbial diversity was observed in obese subjects when compared to those non-obese subjects (Verdam et al., 2013).

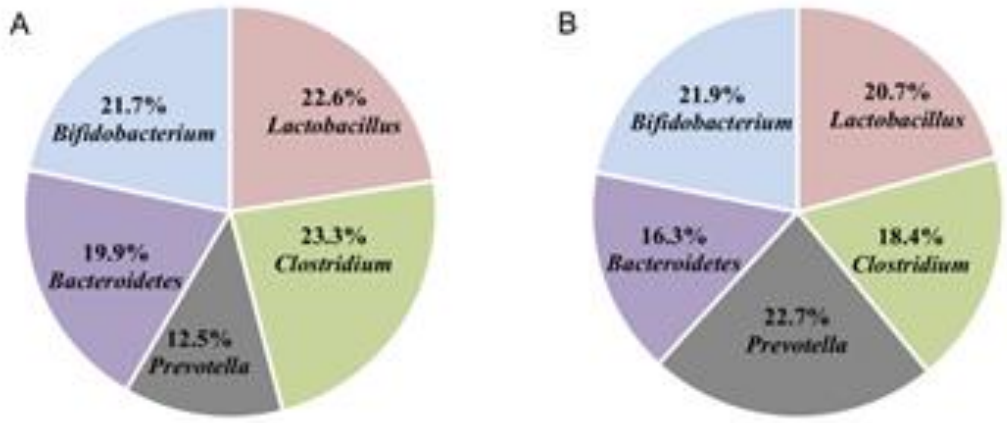


Figure 2. 7 Main types of bacteria isolated from the stool. (A) from obese children (B) from normal children. (Barczynska et al., 2018)

Type 2 Diabetes

Evidence suggested that some *Prevotella* strains present in the gut may improve health by reducing cardiovascular risk and enhancing glucose metabolism (Kovatcheva-Datchary et al., 2015). In addition, it was found that the ratios of Bacteroidetes to Firmicutes and *Bacteroides* to *Prevotella* were higher in Type 2 diabetic patients. These ratios were also positively correlated with the plasma glucose level (Larsen et al., 2010). Furthermore, the level of *Clostridium* species and *Bacteroides caccae,* which belong to opportunistic pathogens, was higher in Chinese type 2 diabetic patients (Qin et al., 2012).

Wheezing and Asthma

A case-control study, nested within the ECUAVIDA birth cohort study, was conducted in rural Ecuador. Cases were those children with wheezing at five years old. When their fecal samples at three months were compared with the healthy group, it was found that the *Bifidobacterium* level was higher while *Streptococcus* sp. was lower in the control group (Figure 2.8). They suggested that atopic wheeze development may be associated with dysbiosis in gut microbiota at three months of age (Arrieta et al., 2018).

Bifidobacterium is a genus that can produce butyrate and plays a role in the gut barrier by protecting against pathogens and diseases. There are varieties of Gram-positive anaerobic bacteria that are non-motile in the genus *Bifidobacterium*. Some strains of the genus *Bifidobacterium* are considered probiotic bacteria, including *Bifidobacterium infantis* and *Bifidobacterium adolescentis.* etc. (Fijan, 2014). It is suggested that *Bifidobacterium* species may help reduce the risk of developing eczema and food allergies (Ruiz et al., 2017).

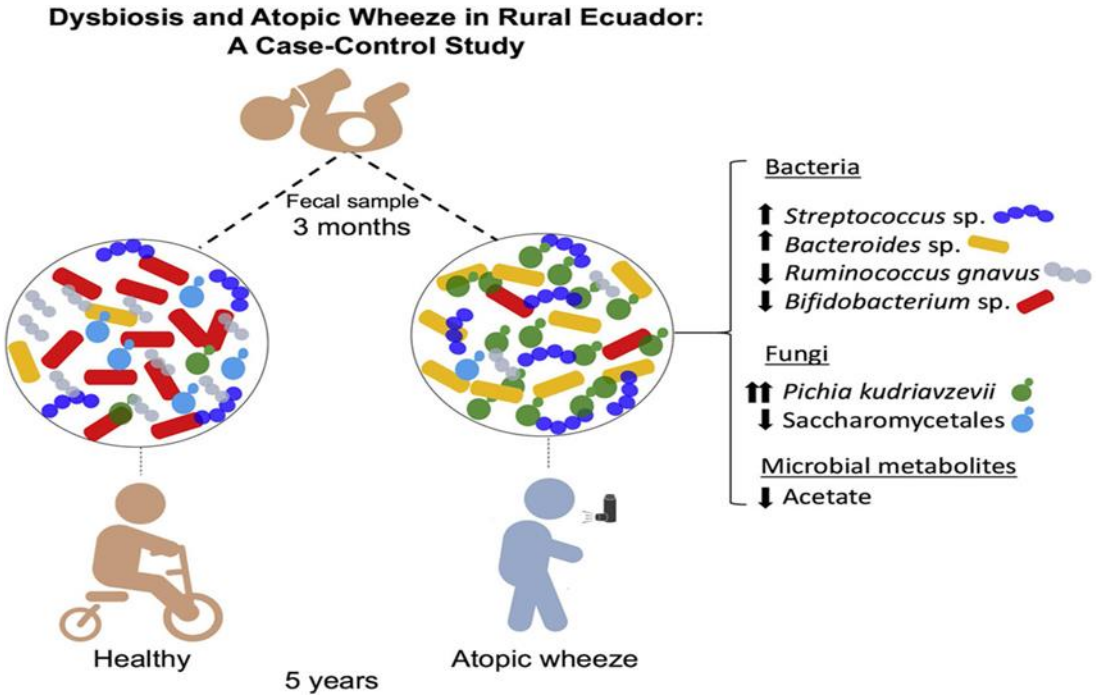


Figure 2.8 Gut microbiota in fecal samples collected in healthy and atopic wheeze groups at three months of age. (Arrieta et al., 2018)

2.7 Factors Affecting the Development of the Gut Microbiota

There are many factors shaping microbial communities throughout the whole prenatal period. During gestation, maternal health status, such as BMI and weight gain during pregnancy, will affect the development of the gut microbiota in infants (Macpherson et al., 2017). Therefore, maternal diet and lifestyle are studied to determine the effects on infants' gut microbiota development. While in the first four months of life, the development is affected by the feeding modes, which are exclusively breastfed, formula fed, or mix-fed. When solid food is introduced, the establishment of the adult–like microbiota will start (Arora & Backhed, 2016). Figure 2.9 summarizes the major and minor factors affecting gut microbiome composition. The following part will discuss some major factors affecting gut microbiota development.

2.7.1Maternal Obesity

Obesity is a risk factor for many chronic diseases. Maternal obesity during the fetal period affects not only the mother's health but also the long-term health of the next generation (Sanli & Kabaran, 2019). One of the proposed mechanisms is that it will affect the gut microbiota development in infants and, thus, their health. A research study found that *Bacteroides* and *Staphylococcus* were higher in infants of overweight mothers than others with normal weight (Collado et al., 2010). Maternal obesity in the lactation period may affect the breast milk content such as leptin, insulin, and glucose level and thus the development of infants' gut microbiota (Fields & Demerath, 2012), and it was associated with a lower level of *Gamma proteobacteria* (Lemas et al., 2016).

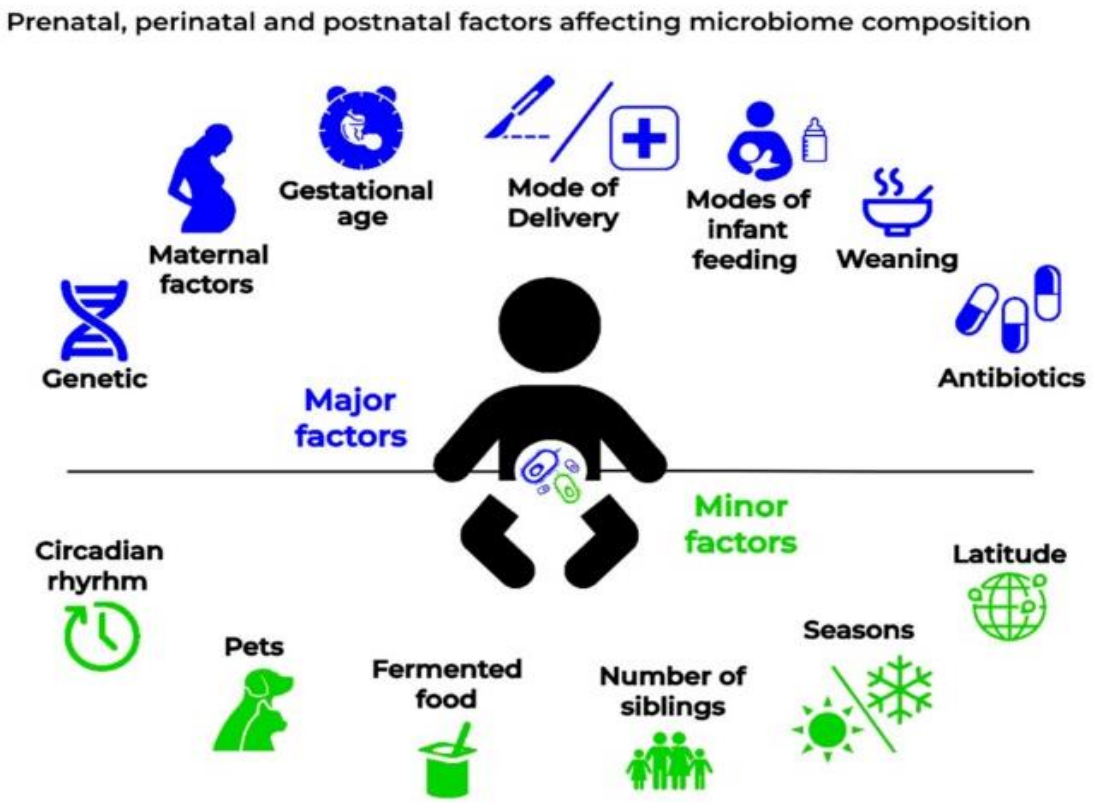


Figure 2. 9 Factors affecting the gut microbiome in infants. (Drago et al., 2019)

2.7.2 Maternal Microbiota during Pregnancy

Even though there is a hypothesis that the womb is a sterile environment for fetus growth, it may not be correct. An early study found that *Staphylococcus* and *Bifidobacterium* were present in the meconium, the first stool from 21 healthy newborns (Jimenez et al., 2008). A research study showed that delivery mode did not affect the bacterial composition in the meconium; the gut microbiome did not differ in babies who are vaginally delivered or delivered through the cesarean section, they proposed that the result implied that colonization of bacteria in the gut started before delivery (Martin et al., 2016).

Due to recent advanced development in the technology of DNA sequencing, technology helps us detect and identify the bacteria in the tissues of mothers 'bodies (Walker et al., 2017). Collado et al. (2016) discovered microbial populations in amniotic fluid and placenta; the most abundant phylum was Proteobacteria, particularly with the species *Enterobacteriaceae,* while the predominant genera were *Enterobacter* and *Escherichia/Shigella*. There was roughly more than 50% bacteria colonization overlap in meconium with amniotic fluid (Ardissone et al., 2014).

However, a study on Chinese pregnant women indicated that cultivable microorganisms could only be found in the placenta but not in the amniotic fluid collected from 64 pregnant women at their 17^{th} – 20^{th} week of gestation (Zhu et al., 2018). Therefore, it is proposed that the microbiota in the fetus may develop in the womb through the placental barrier or consumption of amniotic fluid (Walker et al., 2017). Figure 2.10 shows the proposed mechanisms of the vertical transfer of bacteria from the mother to the fetus. In addition to vertical transmission, maternal microbiota may affect the development of the infant's immune system by those bacteria metabolites. Figure 2.11

shows the overall picture of the proposed mechanisms between maternal microbiota and the immunity development of the infants. The details of the effects of maternal microbiota on breast milk will be discussed in the later sections.

2.7.3 Maternal Diet

Maternal gut microbiota could be one of the sources of bacteria in human milk. Sindi et al. (2021) proposed dietary modulation, which can alter the gut microbiome in mothers and, in turn, influence the human milk and, thus, the infant gut microbiomes. Evidence from some human studies suggested that the maternal diet during pregnancy may affect the gut microbiota of an infant (Chu et al., 2016; Garcia-Mantrana et al., 2020; Lundgren et al., 2018; Nykjaer et al., 2019) through different proposed mechanisms. Sindi et al. (2021) also raised that diet during lactation should also influence the infant gut microbiota; however, there is limited research in this area. Details refer to section 2.9.

2.7.4 Mode of Delivery

There are two major ways of delivery: vaginal delivery and cesarean section. Mode of delivery is one of the critical factors determining the establishment and development of the infant's gut microbiota. It is found that the gut microbiota was different in infants delivered by cesarean section and vaginal delivery (Arora & Backhed, 2016).

PCoA in a study of stool samples from birth to 6 months old indicated that microbiota in two delivery modes differed significantly (Yang et al., 2019). The total diversity of gut microbiota was lower in the first week of life in infants delivered by cesarean section when compared with vaginal-delivered infants (Rutayisire et al., 2016). A very early study conducted in 1999 indicated that *Bifidobacterium* was significantly higher

in infants delivered by vaginal delivery at the first week of life. There was more colonization of *Bacteroides* in vaginal-delivered infants at 10 and 30 days after birth (Gronlund et al., 1999). Another study also showed that *Lactobacilli* colonization was higher in vaginal-delivered infants at the first week of life in healthy Greek neonates (Mitsou et al., 2008). In addition, *Bacteroides* and *Escherichia coli* levels were higher in the guts of vaginal-delivered infants (Mueller et al., 2015). In contrast, the *Clostridium* genus was more abundant in infants delivered by cesarean section when compared with the vaginal-delivered infants at 21 days (Hesla et al., 2014).

A review study tried to combine the abundance level in different studies, and the result is shown in Figure 2.12. Overall, there was a lower level of colonization of *Bacteroides*, *Lactobacillus,* and *Bifidobacterium* in babies delivered by cesarean section. At the same time, *Clostridium* difficile and *Staphylococcus* were higher than babies vaginally delivered in the early stage of life (Moore & Townsend, 2019). Since development is affected by many factors after birth, some studies suggested the differences between the patterns of the two groups were not that much at the age of 6 months. Except, higher the level of *Bacteroides*, and lower level of *Clostridium* in those were naturally delivered (Rutayisire et al., 2016).

For those babies who were vaginally delivered, their microbiota composition resembled the microbiota of the vagina in mothers (Rinninella et al., 2019). While the microbiota in babies born by cesarean section was more similar to the hospital environment and mothers' skin (Azad et al., 2013). One of the reasons for a lower amount of *Bifidobacterium* and *Bacteroides* and higher *Clostridia* colonization in the infants delivered by cesarean section may be due to the antibiotic use before, during, and after the delivery process (Rutayisire et al., 2016).

2.7.5 Mode of Early Feeding

It was suggested that feeding an infant with breast milk exclusively in their early life is one of the factors modifying the gut microbiota in infants (Serino et al., 2017). *Bifidobacterium* and *Lactobacillus* in feces of Breast-fed infants were significantly higher than in formula-fed infants, while *Bacteroides* and *Clostridium* level was higher in the formula-fed group (Bezirtzoglou et al., 2011; Fallani et al., 2010; Jost et al., 2015). Figure 2.13 summarizes the effect of different factors on infant gut microbiota development.

In addition, a systematic review conducted by Ho et al. (2018) concluded that seven studies showed that the alpha diversity in the exclusively breastfed group was lower than the others. It has been proposed that the effects of breast milk on infants' gut microbiota are due to the presence of both prebiotics and probiotic in the milk. Prebiotics is the HMO, while probiotics are the milk microbiota present in human milk (Moossavi et al., 2018). Milk microbiota can be transferred vertically from mothers to infants through breast milk (Solis et al., 2010), while HMO, a nutrient source, can play a crucial role in shaping the growth and function of microorganisms in infants' gut selectively (Milani et al., 2017).

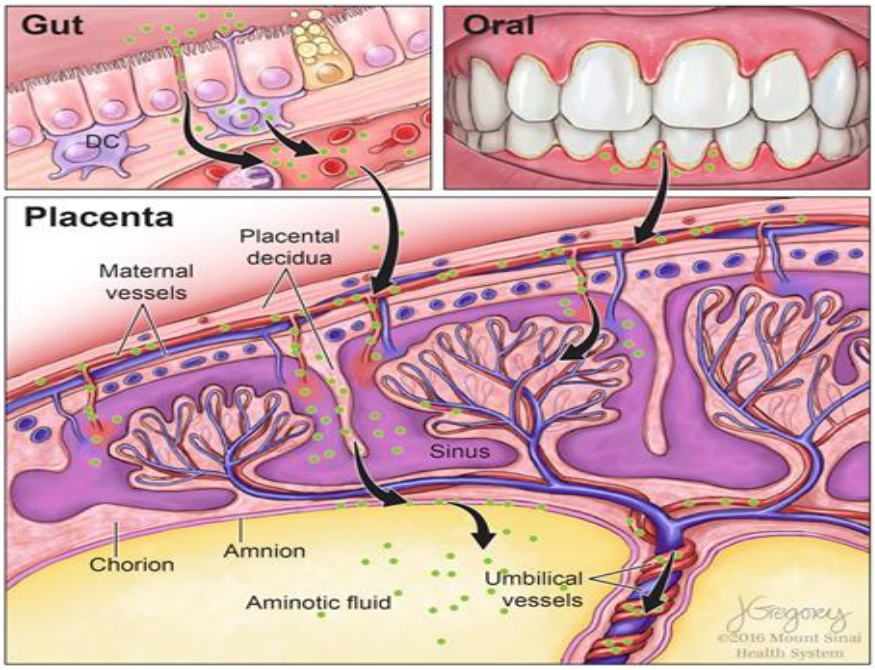


Figure 2. 10 Proposed route of bacteria transmission to the fetus from the mother. (Walker et al., 2017)

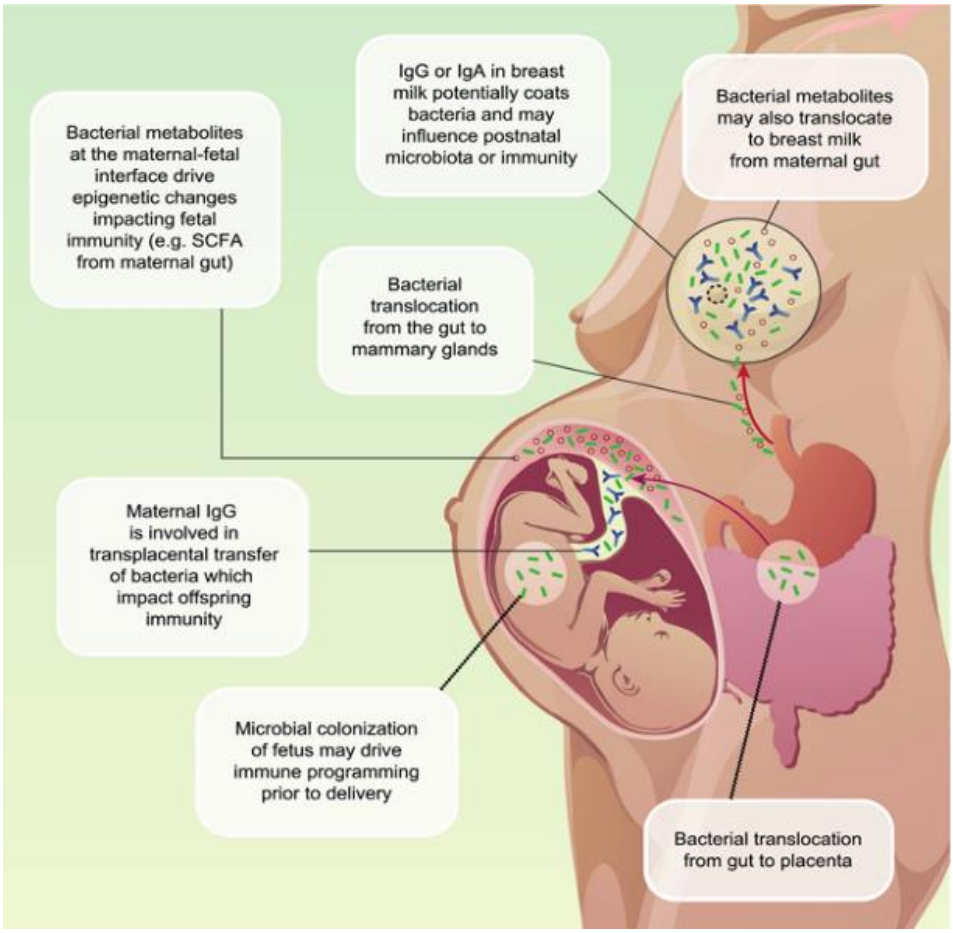


Figure 2. 11 Proposed mechanism of the effects of maternal microbiota on the immunity of infants. (Nyangahu & Jaspan, 2019)

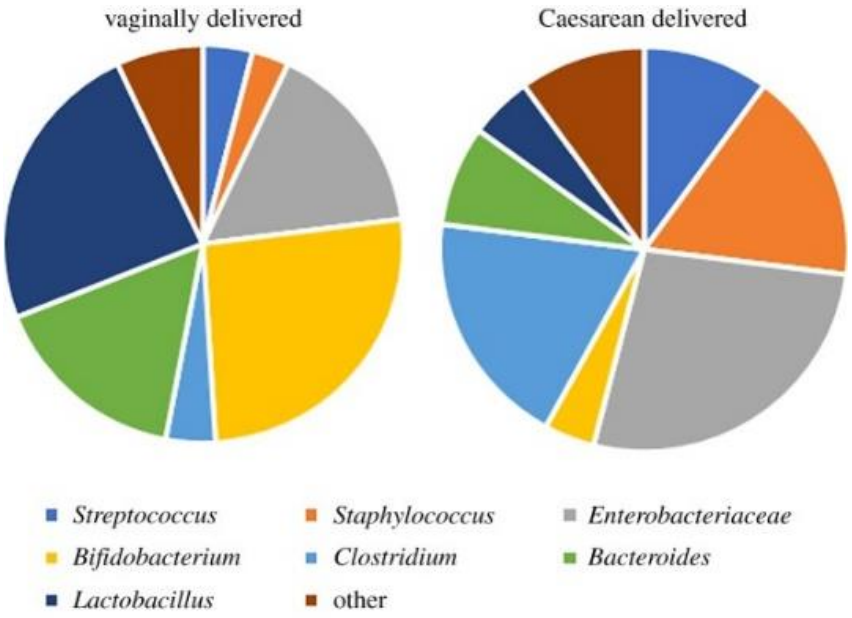


Figure 2. 12 Bacteria in vaginally delivered and Caesarean-delivered infants. (Moore & Townsend, 2019)

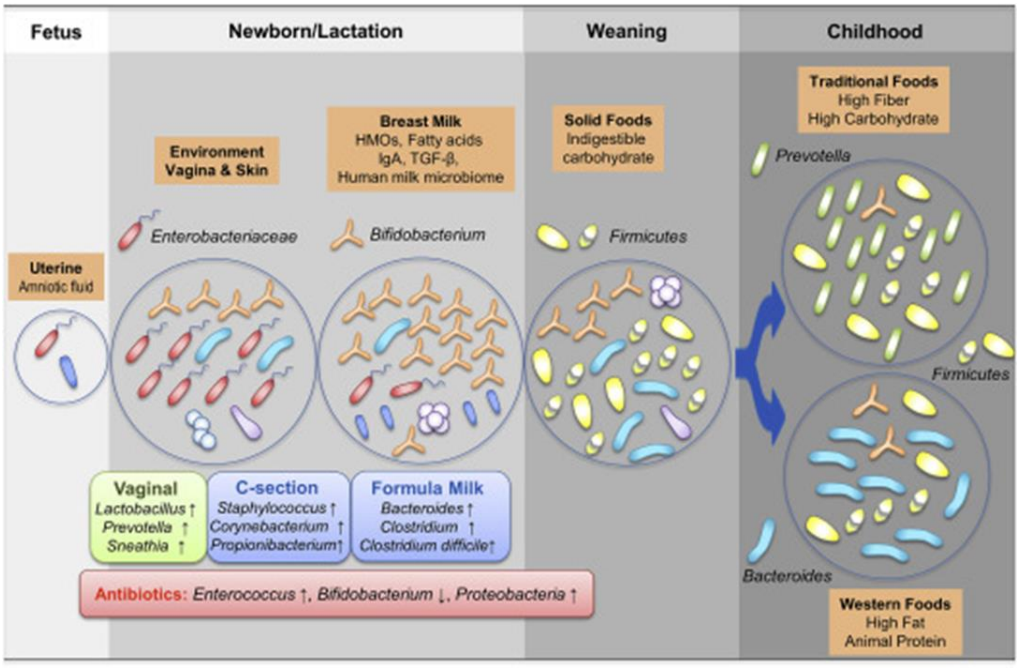


Figure 2. 13 Factors affecting the gut microbiota in infants. (Tanaka & Nakayama, 2017)

2.8 Breast Milk, Human Milk Oligosaccharides, Microbiota in Milk, and

Infants' Gut

Milk is the first source of nutrients for infants; they are either fed with breast milk or infant formula. The World Health Organization (WHO) recommends exclusively for the first six months, followed by solid food introduction with continued breastfeeding for up to 2 years old or after (WHO, 2017). Human milk is the gold standard for infants because it contains a suitable amount of nutrients for growth. More evidence showed that it may contain beneficial bacteria, which can lower the chance of getting the disease in the baby (Lyons et al., 2020).

2.8.1 Composition of Breast Milk

Jenness (1979) discovered that the percentage of macronutrients in mature human milk was roughly 3% - 5% fat, 0.8% - 0.9% protein, and carbohydrate around 7%. The energy content is approximately 70 kcal/100ml. The protein content of human milk is comparatively low when compared with cow's milk which is roughly 3.5 % (Boquien, 2018). Within that 7 % carbohydrates, 1 to 2.4% were oligosaccharides (Boquien, 2018). Therefore, apart from the major component's lactose and lipids, oligosaccharides are the third most abundant component in breast milk. Oligosaccharides are polymers consisting of 3-10 monosaccharides. The basic structures of the human milk oligosaccharides are composed of 5 basic monosaccharides: glucose, galactose, N-acetylglucosamine, fucose, and sialic acid with N-acetylneuraminic acid (Bode, 2012). One liter of mature human milk is estimated to contain 5 to 20 g of these complex sugars (Milani et al., 2017). Figure 2.14 shows the composition of human milk.

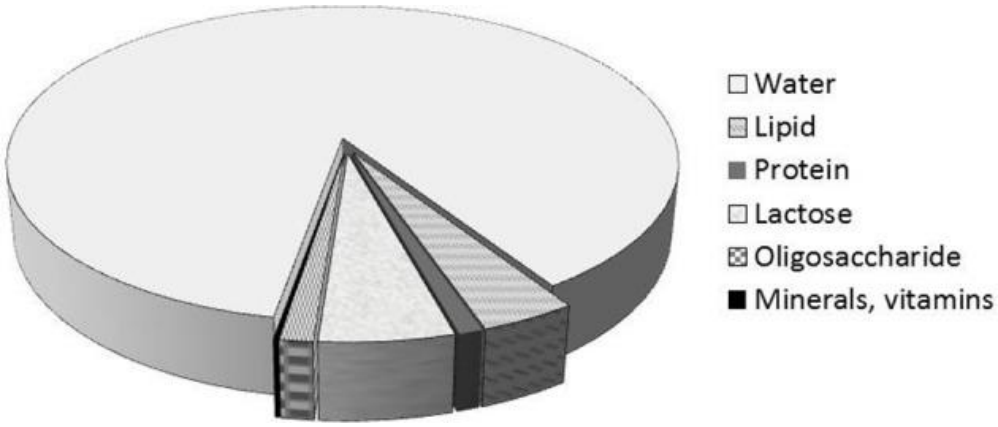


Figure 2. 14 Composition of human milk. (Boquien, 2018)

2.8.2 Oligosaccharides in Breast Milk

Human milk mainly contains lactose, lipids, and human milk oligosaccharides (HMO), the third most abundant group in human milk (Kunz et al., 2000). Infants do not have the specific enzyme to break down the HMO present in human milk, and the HMO will then arrive in the large intestine and readily be used by the gut microbiota (Jost et al., 2015). Over 200 different types of HMO have been identified (Plaza-Diaz et al., 2018). Figure 2.15 shows the basic structure of the HMO.

However, HMO are in different complex structures composed of 3-20 units of sugar but in different isomer forms (Totten et al., 2012). There are more than hundreds of different HMO without a standard reference. Therefore, it is challenging for researchers to measure the absolute quantity of HMO (Xu et al., 2017).

Xu et al. (2017) tried to set up a way to measure the absolute quantitation of HMO using ultraperformance liquid chromatography triple quadrupole-mass spectroscopy (UPLC/QqQ-MS) in multiple reaction monitoring (MRM) mode. Hydrophilic interaction liquid chromatography (HILIC)- UPLC was used to separate the HMO in the milk. Then QqQ-MS was used to detect and quantify the HMO present.

Every woman may synthesize a different set of oligosaccharides in breast milk (Kobata, 2010). Lactating mother with a functional alpha-L-fucosyltransferase 2 (FUT2) enzyme is called the secretor phenotype; the human milk composition will be different compared to non-secretors (Kunz et al., 2017). The total human milk oligosaccharides concentration has been shown to be significantly higher in “secretor” milk when compared with “non-secretor” milk. The concentration difference was mainly due to the high concentration of 2'-Fucosyllactose (2'FL), Lacto-N-fucopentaose I (LNFP I),

and Lacto-N-difucohexaose I (LNDFH I) in “secretor” milk, while they were not found in “non-secretor” milk. However, core oligosaccharide Lacto-N-tetraose (LNT) in breast milk was higher in non-secretor milk when compared with the secretor one. The “non-secretor” lactating women with the Lewis blood group (a+b-) have roughly 35-45% less human milk oligosaccharides when compared with breast milk from the Lewis blood group (a-b+) secretor (Kunz et al., 2017). It showed that higher alpha-L-fucosyloligosaccharides present in human milk may have a lower risk of diarrhea in infants (Newburg et al., 2004). Since alpha 1,2 fucosylated HMO were higher in milk from secretor mothers, a study proposed that the amount of alpha 1,2 fucosylated HMO can be used as a specific marker to check the secretor status of mothers (Xu et al., 2017). The secretion of HMO in mothers with different genes is summarized in Figure 2.16

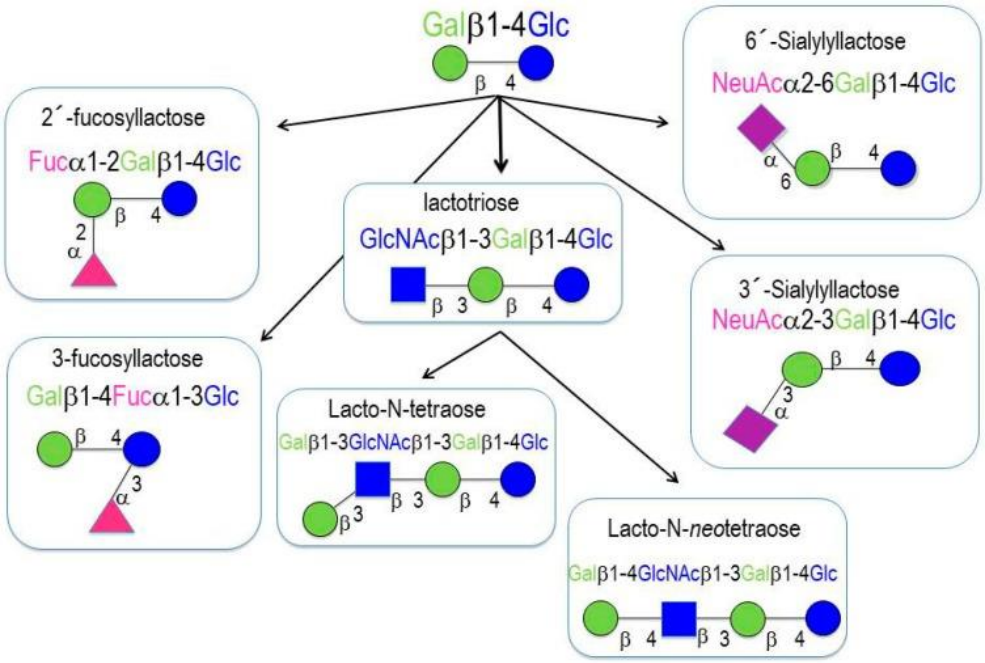


Figure 2. 15 Basic structure of HMO in human milk. (Plaza-Diaz et al., 2018)

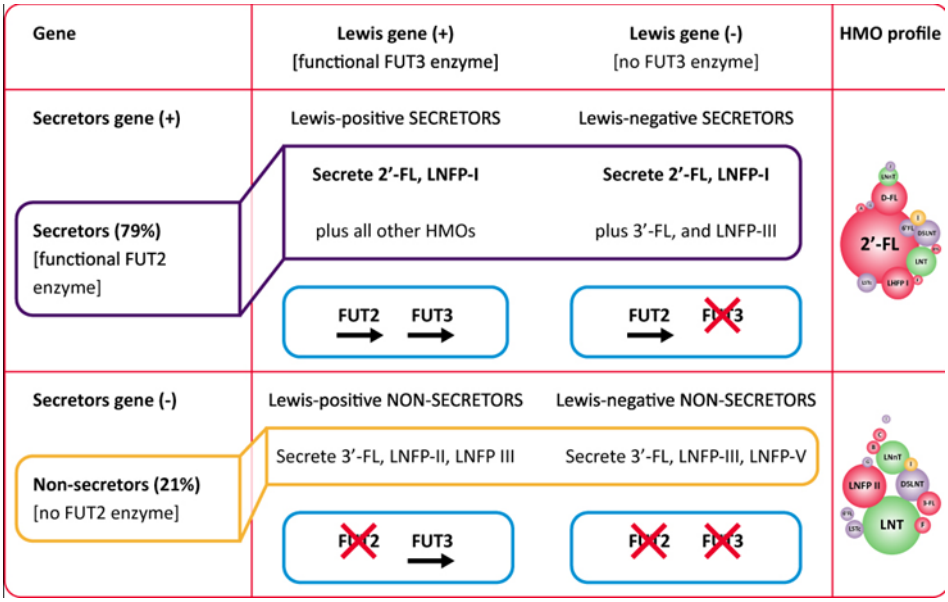


Figure 2.16 HMO secretion in mothers with different genetic backgrounds. (Sekerel et al., 2021)

2.8.3 Microbiota in Breast Milk

Human milk was considered to be free of germs previously; however, it has been found that it may be wrong because research recently can isolate and identify the microbiota in milk (Arrieta et al., 2014; Drago et al., 2019). More than 200 species of bacteria have been isolated from breast milk (Jeurink et al., 2013). It has been proposed that the origins of the bacteria in milk are from babies' oral cavities, mothers' skin, and the lactiferous ducts in mothers or through the entero-mammary pathway (Corona-Cervantes et al., 2020). The entero-mammary pathway hypothesizes that the maternal microbiota can be translocated to the mammary glands via lymphatic circulation. Maternal gut microbiota is also considered one of the sources of human milk microbiota. It is also proposed that they can pass through the intestinal epithelial barrier and enter lymphatic circulation (Fitzstevens et al., 2017).

Streptococcus and *Staphylococcus* were the predominant genera found in breast milk. *Lactobacillus*, *Bifidobacterium*, *Clostridium,* and *Bacteroides* were also identified in the milk (Fitzstevens et al., 2017). Different research studies also try to investigate the microbiota in the milk of different cohorts/ countries. Figure 2.17 shows the result of milk microbiota in different studies. In a research study conducted to investigate the milk microbiota in Chinese lactating mothers, the result was consistent with other cohorts in that *Streptococcus* and *Staphylococcus* dominated the milk. They also could observe *Bifidobacterium* and *Lactobacillus* in some samples; however, the level was low (Sakwinska et al., 2016).

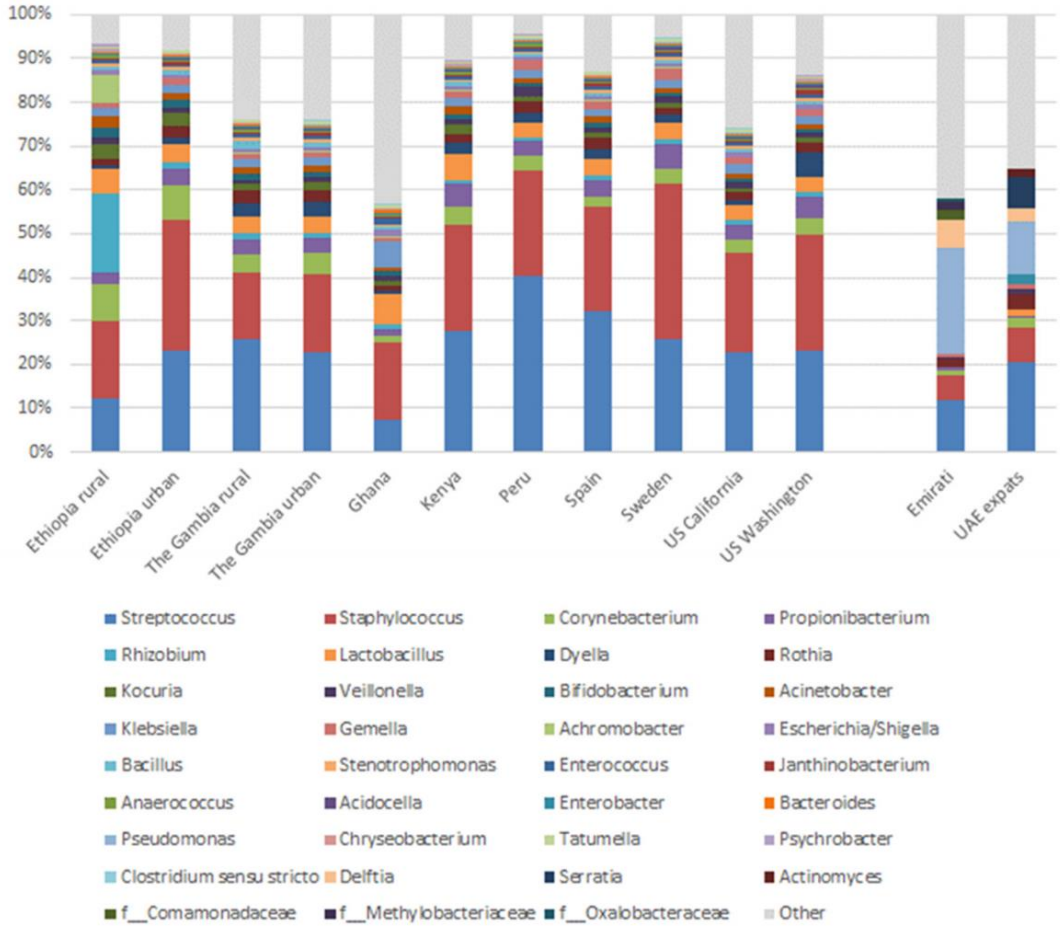


Figure 2. 17 10 Most abundance genera level in the milk of different cohort studies. (Corona-Cervantes et al., 2020)

2.8.4 HMO and Milk Microbiota

The HMO in milk were associated with the microbiota in milk, fucosyllacto-N-hexaose (FLNH) and lacto-N-hexaose (LNH) were associated with microbiota richness, while lacto-N-fucopentaose I (LNFP I) (was negatively associated with microbiota diversity. Only a weak association can be identified between Salylated/non-fucosylated HMO correlated with *Prevotella* (Moossavi et al., 2019).

2.8.5 HMO and Gut Microbiota

One of the explanations for higher *Bifidobacterium* in babies fed with exclusive breast milk could be due to the presence of HMO which are fermented by the colonic bacteria, mainly by *Bifidobacterium*. This can decrease the pH in the intestinal environment and thus protect the infant from other pathogenic organisms (Bode, 2009). Some manufacturers may also add oligosaccharides into the infant formula, such as Fructooligosaccharide (FOS) and Galactooligosaccharide (GOS), which are shown to promote the growth of *Bifidobacterium spp* (Haarman & Knol, 2005). However, it was suggested that these two oligosaccharides are less complex than the human breast milk oligosaccharides and lack anti-adhesive or immunomodulation effects (Jost et al., 2015).

The gut microbiota in infants was influenced by lacto-N-fucopentaose III (LNFP III) concentration in milk at 2-week, 3'- sialyllactose (3'SL) in milk at six weeks, and LNH in milk at 12 weeks. However, the correlation between individual HMO and each bacterium in the gut was weak, including *Bifidobacterium*. When analyzing the HMO in stool samples, they were negatively correlated with the relative abundance of genera *Bifidobacterium*, *Escherichia-Shigella,* and *Bacteroides* (Borewicz et al., 2020).

2.8.6 Milk Microbiota and Infants' Gut Microbiota

Bacteria cells, including *Staphylococcus*, *Bifidobacterium,* and *Lactobacillus* in breast milk, could be the inoculum source for the infant's gut microbiota. These bacteria may directly transfer vertically from the mother to the neonate through breastfeeding (Solis et al., 2010). It was suggested that the presence of lactic acid bacteria in breast milk was one explanation for higher *Bifidobacterium* in babies fed with exclusive breast milk (Martin et al., 2003).

However, it was found that the abundance of microbiota in milk was not the same as the one in infants' stool. The alpha diversity in milk was higher than in infants' stool samples (Corona-Cervantes et al., 2020). Ten pairs of lactating mothers and their infants were recruited. Concerning phyla level at week 1, human milk was dominated by Proteobacteria, Firmicutes, and Bacteroidetes. In contrast, higher relative abundances of Firmicutes, Actinobacteria, and a lower percentage of Proteobacteria and Bacteroidetes, while at week 3, the dominant phylum in milk was Firmicutes (Murphy et al., 2017).

2.9 Maternal Diet during Pregnancy and Lactation

Pregnancy and lactation are both critical periods for establishing the risk of long-term chronic diseases in the next generation (Barker, 2007). Nutrition plays a significant role in these periods. During pregnancy, the nutritional status of mothers could affect the pregnancy outcomes and perinatal outcomes. During lactation, a healthy diet is one of the ways to support optimal health for infants via human milk, which is personalized nutrition for babies (Marshall et al., 2022).

It has been proposed that the maternal diet can affect the gut microbiome of mothers, for example, the fiber intake, which can influence short-chain fatty acid (SFCA) production. The gut microbiota in the mother may directly transfer through milk via an entero-mammary pathway to the baby. This influences the infant's gut microbiota development either through the direct transfer of bacteria or the SCFAs and HMO in breast milk (Sindi et al., 2021). The potential effects of maternal diet on infant gut microbiota are summarized in Figure 2.18.

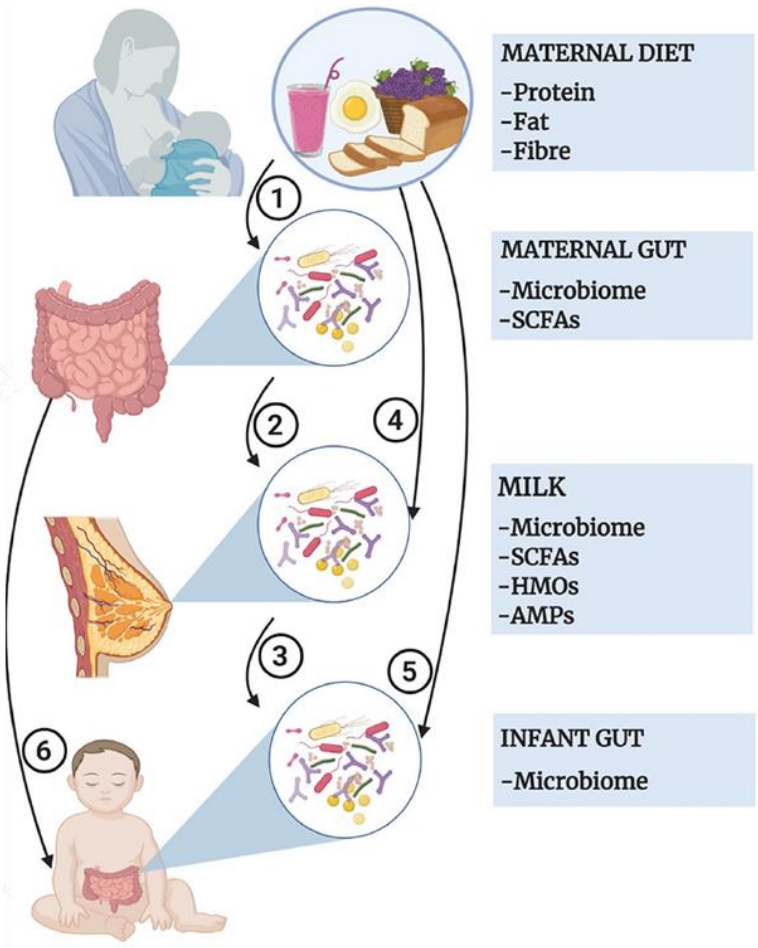


Figure 2.18 Potential effects of maternal diet on the maternal gut, milk, and infant gut microbiota. (Sindi et al., 2021)

2.9.1 Recommended Pregnancy Diet

According to the Department of Health (DH), HKSAR, the body needs slightly more calories, similar to an extra piece of whole bread or a cup of low-fat milk a day. Then the body needs more calories when entering the second and third trimesters by increasing the variety of high-quality food. The recommendation of several serving recommended by the on different food groups is as follows (DH, 2022):

Food Group	Serving per day		Example of a serving
	1st trimester	2nd-3rd trimester	
Grains	3 – 4	3.5 – 5	1 bowl of rice 2 slices of bread
Vegetables	Three or more	4 – 5	1/2 bowl of cooked leafy vegetables 1 bowl of uncooked vegetables
Fruits	2 or more	2 – 3	1 medium size fruit such as orange/apple of lady's fist 2 kiwi fruits
Meat, fish, egg, and alternative	5 – 6	5 – 7	40 g raw meat/fish 30 g cooked meat/fish 1 egg
Milk and alternatives	1 – 2	2	1 cup of milk 2 slices of cheese

According to MyPlate, the recommendation is as follows (USDA, 2016b):

Food Group	Per day		Example of an ounce / a cup
	1st trimester	2nd-3rd trimester	
Grains	6 ounces	8 ounces	1/2 bowl of rice / 1 slice of bread
Vegetables	2.5 cups	3 cups	1 bowl of vegetables/ 2 bowls of leafy vegetables
Fruits	2 cups	2 cups	1 cup of fruit/ 2-3 kiwi fruits/ 1 small apple
Protein	5.5 ounces	6.5 ounces	1-ounce meat / 1 egg
Dairy	3 cups	3 cups	1 cup of milk 2 slices of cheese

2.9.2 Maternal Diet during Pregnancy in Asia

Some studies were conducted to examine the diet in China (Zhang et al., 2020) and South Asia (Imai et al., 2021). Imai et al. (2021) found out that the average energy intake of pregnant women was 1680kcal, while Zhang et al. (2020) found that the average energy intake of the third trimester was 2065kcal and higher meat, dairy, and dairy products, fish and shrimp, and soybeans consumption in the 2nd and third trimesters compared to the first trimester. Limited data related to pregnancy diet in Hong Kong could be found. The dietary intake of ethnic Chinese pregnant women in Hong Kong was analyzed, but the study was published more than 20 years ago. Wong et al. (1997) demonstrated that the meat and its substitute intake was significantly higher than the recommended intake, while vegetable intakes were lower. Another research study on early pregnancy diet in HK was published recently by Tsoi et al. (2022). The average energy intake was 1938kcal, and they found that 99% of the subjects had excessive sodium intake and only 2.6% could meet the recommended fiber intake.

2.9.3 Maternal Overnutrition during Pregnancy and Infant Health

A woman with a normal BMI who is physically active, has a healthy diet, do not smoke will have a healthy pregnancy outcome (Koletzko et al., 2014; Koletzko et al., 2019). Research studies indicated that maternal obesity, excessive gestation weight gain, and overnutrition in pregnancy were associated with fat mass in children and higher cardiovascular risk (Chen et al., 2016; Koletzko et al., 2014; Toemen et al., 2016).

2.9.4 Maternal Undernutrition during Pregnancy and Infant Health

Maternal undernutrition is more common in developing countries (Zahangir et al., 2017). It can lead to a higher risk of stillbirth, neonatal morbidity, long-term growth

deficits, and neurocognitive development (Bilal et al., 2022). Women with undernutrition during pregnancy had a higher chance of delivering infants of low birth weight (Bilal et al., 2022). Research indicated that babies with low birth weight showed rapid catch-up growth after birth and experienced significant weight gain. The risk for obesity, type two diabetes, and cardiovascular diseases in adulthood were higher (Adair & Cole, 2003; Popkin et al., 1996).

2.9.5 Maternal Diet during Pregnancy and Human Milk Microbiota

One study conducted by Padilha et al. (2019), which assessed the pregnancy diet through Food Frequency Questionnaire and collected breast milk from mothers, found that the *Staphylococcus* spp. in human milk was positively correlated with vitamin C intake in the maternal diet during pregnancy. Another study also showed that the microbiota in breast milk was associated with maternal diet. *Staphylococcus* and *Bifidobacterium* were associated with carbohydrate intake, and the *Streptococcus* genus was associated with intakes of EPA and Docosapentaenoic acid (Cortes-Macias et al., 2021).

2.9.6 Maternal Diet during Pregnancy and Infant Gut Microbiota

Lungren et al. (2018) conducted a food frequency questionnaire to assess the maternal diet during pregnancy and collected the infants' stool samples at six weeks postpartum. They found that maternal dairy intake was positively associated with the genus *Staphylococcus* and species *Clostridium neonatale* and *C. butyricum*. While seafood intake was positively associated with the genus *Streptococcus* including the species *Streptococcus* agalactiae but was negatively associated with the species *Bacteroides uniformis*. Fruit intake was positively associated with the Family Clostridiaceae while negatively associated with the genus *Bifidobacterium* (Lundgren et al., 2018).

It has been suggested that fish consumption is important during pregnancy due to its high omega-3 fatty acids, including EPA and DHA, for the neurodevelopment of infants (Nykjaer et al., 2019). A study identified 3 distinct profiles in the fecal microbiota of infants: *Bifidobacterium* dominant profile, *Enterobacter*-dominant profile, and *Escherichia* dominant profile. Moreover, it was found that the RR ratio was higher for *Bifidobacterium* dominant profile in the infant's stool sample when mothers met the recommendation of fish consumption during pregnancy, which is 4 oz per week (Simione et al., 2020). Garcia-Mantrana et al. (2020) showed an association with maternal diet during pregnancy. Higher maternal fiber and vegetable protein intake was negatively correlated with infant gut Bacteroidetes, while high animal protein intake during pregnancy was positively correlated with infant gut Acinetobacteria. Besides the association with the intake of different food groups, a study indicated that a maternal diet with high-fat level was associated with a trend of the lower level of Bacteroides in the infant's gut at the first stool sample and also the stool at six weeks of age (Chu et al., 2016).

2.9.7 Maternal Diet during Pregnancy and HMO

Azad et al. (2018) conducted a large population-based cohort study and showed no associations with HMO concentrations in breast milk collected at 3–4 months post-partum with maternal dietary nutrient intake during pregnancy. Only total energy intake was positively correlated with LNT and difucosyllacto-N-tetrose (DFLNT).

2.9.8 Recommended Lactating Diet

Mothers need to have a healthy balanced diet to sustain the health of mothers themselves as well as the infants via supplying the nutrient in the breast milk. The number of serving recommended by the DH, HKSAR on different food groups is as follows (DH, 2022):

Food Group	Serving per day	Example of a serving
Grains	4 – 5	1 bowl of rice 2 slices of bread
Vegetables	4 – 5	1/2 bowl of cooked leafy vegetables 1 bowl uncooked vegetables
Fruits	3	1 medium size fruit such as orange/apple of lady's fist 2 kiwi fruits
Meat, fish, egg, and alternative	6 – 7	40 g raw meat/fish 30 g cooked meat/fish 1 egg
Milk and alternatives	2	1 cup of milk 2 slices of cheese

According to MyPlate, the recommendation is as follows (USDA, 2016a):

Food Group	Per day		Example of an ounce / a cup
	Exclusively breastfeeding	Mix feeding	
Grains	8 ounces	6 ounces	1/2 bowl of rice 1 slice of bread
Vegetables	3 cups	2.5 cups	1 bowl of vegetables 2 bowls of leafy vegetables
Fruits	2 cups	2 cups	1 cup of fruit
Protein	6.5 ounces	5.5 ounces	1-ounce meat 1 egg
Dairy	3 cups	3 cups	1 cup of milk 2 slices of cheese

2.9.9 Maternal Diet during Lactation in Asia

A study was conducted to investigate the lactating diet of women in 13 provinces in China, such as Shanghai, Beijing, and Guangdong (Ding et al., 2020). The result suggested that the energy intake ranged from 1327.45 to 2085.97 kcal; 83.8% of subjects was lower than the estimated energy reference. In addition, the consumption of vegetables, fruits, fish, seafood, and dairy products was lower than the recommendation. A study which was investigating the association between maternal diet and human milk composition as well as infant growth in Japan during the first month of life indicated that the average energy intake during lactation was 1594.5 kcal ± 448.7 (SD) (Komatsu et al., 2023). In Hong Kong, there was a study on vitamin A content in breast milk and maternal diet during lactation. Lu et al. (2022) reported that the average energy intake of lactating mothers was 2393.1 kcal.

2.9.10 Maternal Diet during Lactation and Nutrient Composition in Human Milk

Limited information could be found in this research area. A systematic review conducted on the effects of diet on human milk composition showed that three studies supported the link between high fish consumption and high docosahexaenoic acid in breast milk, while two studies reported a positive correlation between dietary vitamin C and milk concentrations of this vitamin (Bravi et al., 2016).

2.9.11 Maternal Diet during Lactation and Human Milk Microbiota

LeMay-Nedjelski et al. (2021) reported a negative association between maternal fiber intake during lactation with *Streptococcus* in human milk. Maternal fat intake during lactation was also associated with human milk microbiota. High monounsaturated fat intake was positively correlated with the genus *Acinetobacter and Gemella,* while the polyunsaturated fat intake was negatively correlated with the genus *Acinetobacter.*

However, more than 1/3 of the subjects in this study were diagnosed with gestational diabetes or impaired glucose intolerance, which may affect the result (Sindi et al., 2021). Another study showed that maternal polyunsaturated fat and linoleic fatty acid intake during lactation was positively associated with genus *Bifidobacterium* in human milk, while the vitamin B1, B2, and folate intake were negatively correlated with genus *Enterococcus* in milk (Padilha et al., 2019).

2.9.12 Maternal Diet during Lactation and HMO in Breast Milk

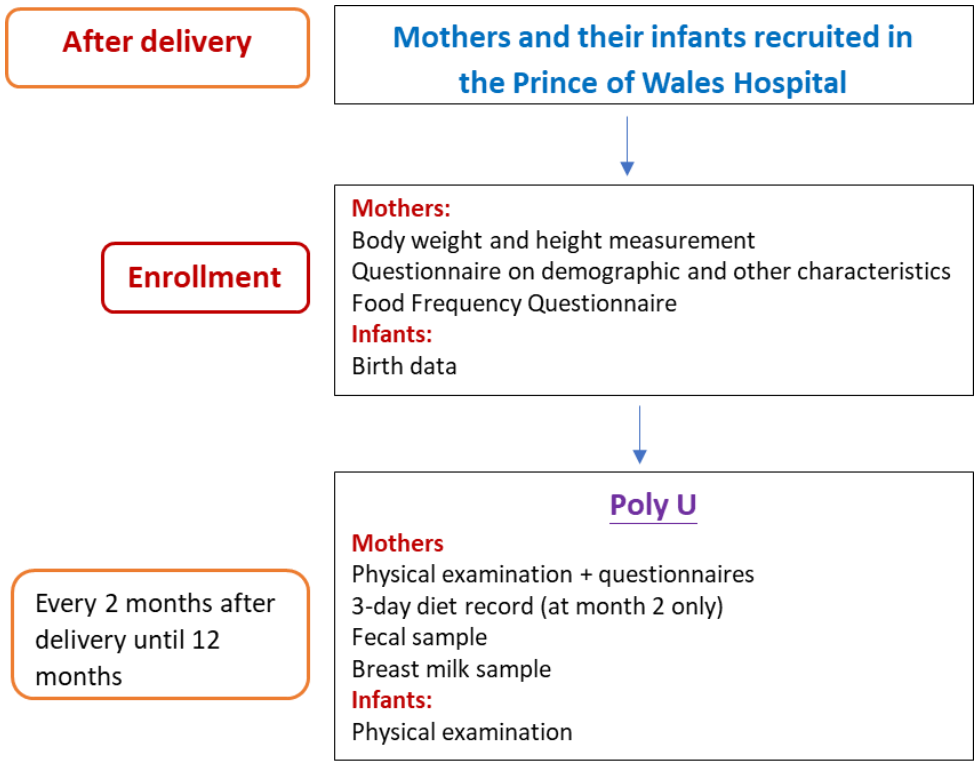
A study conducted in China found that vitamin A, vitamin C, and vegetable intake during lactation were positive predictors of 3-Fucosyllactose (3FL), while vitamin B1 and B2 were positively associated with 2'FL and the sum of 2'FL and 3FL. Milk and lactose intake were positively associated with LNT and the sum of LNT and lacto-N-neotetraose (LNnT) (Li et al., 2022).

Maternal macronutrient intake during lactation may affect the biosynthesis of HMO. Quin et al. (2020) reported that some sulfonated HMO were positively correlated with maternal intake of monounsaturated and polyunsaturated fats while negatively associated with saturated fat intake, particularly in secretor mothers. This study also showed that fruit intake was positively correlated with some HMO. In addition, whole grain intake during lactation was positively correlated with FLNH; however, no other correlation could be observed in the same study (Quin et al., 2020). Some studies focus on the relationship between food group intake or macronutrient intake during lactation and HMO in human milk, the information on micronutrient intake in the maternal diet was limited. One of the studies conducted in 2013 showed that higher vitamin A during lactation increased the sialic acid in the milk sample (Qiao et al., 2013).

Chapter 3 Methodology

This research study mainly involved a cohort study to collect maternal diet information together with breast milk samples and infant fecal samples at month 2, 4, 6, 8, 10 and 12 and a cross-sectional study to collect more infant fecal samples at month 2 and 4 for analyzing the effect of early feeding practice on development of infant gut microbiota.

Flowchart of cohort study:



3.1 Analysis of Maternal Diet during Pregnancy

3.1.1 Recruitment of Subjects

Subject recruitment took place in health centers in both public and private health sectors. Subjects were recruited using an opportunity sampling method and screened by our research staff to check the eligibility. Mothers should fulfill the following inclusion criteria: Hong Kong residents having resided in Hong Kong for a continuous period of not less than 18 months; Had normal pre-pregnant BMI (i.e,. 18.5-22.9); They have not

participated concurrently in any clinical trial or study; With no complicated pregnancy such as preeclampsia and gestational diabetes; No special dietary restrictions for examples gluten-free diets, vegan or any restrictions due to food allergies. For eligible mothers, they were explained with the study details and informed written consent was obtained from all subjects (Appendix.1). Ethics approval was obtained from the Human Subjects Ethics Sub-Committee (reference number: HSEARS20180123009-03).

3.1.2 Data Collection

Demographic information including the mother's age, occupation, family income, smoking status, alcohol use, antibiotic use, BMI and weight gain during pregnancy, were collected using standardized questionnaires (Appendix.2).

3.1.3 Nutritional Assessment

Dietary records were collected using a Food Frequency Questionnaire (FFQ) on the regular diet during the pregnancy. A validated FFQ (Appendix 3) was modified and used to determine their average energy intake and dietary intake of specific nutrients in the last trimester of pregnancy(Woo et al., 1997). The questionnaire comprised 8 major food categories with a total of 160 food items that included vegetables, fruits, legumes, grains, meat, fish and seafood, eggs, dairy products, and beverages. Mothers reported their average frequency of consumption of foods and drinks during the last trimester of pregnancy. The reporting of the portion consumed was facilitated by providing the participants with food photographs (the weight of 100g of most frequently consumed foods). Based on the category of consumption frequency and the portion size consumed, the average amounts for each food were calculated into g/day. Then the nutrient intakes per day were calculated using the ESHA Food Processor.

3.2 Analysis of Maternal Diet during Lactation

3.2.1 Recruitment of Subjects

Subject recruitment took place in health centers in both public and private health sectors, and platforms provided by organizations promoting breast feeding include MaMa Milk Baby Alliance, Leche League, and Hong Kong Breastfeeding Mothers' Association. Subjects were recruited using an opportunity sampling method and screened by our research staff to check the eligibility.

Mothers should fulfill the following inclusion criteria: Hong Kong residents having resided in Hong Kong for a continuous period of not less than 18 months; Had normal pre-pregnant BMI (i.e., 18.5-22.9) and weight gain during pregnancy (i.e., 11-16.4kg); Deliver at full term (>37 gestation weeks); Give birth to singleton infant within the normal birth weight of > 2500g. They have not participated concurrently in any clinical trial or study; With no complicated pregnancy such as preeclampsia and gestational diabetes; No special dietary restrictions, for example gluten-free diets, vegan or any restrictions due to food allergies. An informed consent form was signed for eligible mothers after receiving the detailed explanation of the study protocol and the study information sheet (Appendix 1). Ethics approval was obtained from the Human Subjects Ethics Sub-Committee (reference number: HSEARS20180123009-03).

3.2.2 Data Collection

Demographic information, including mother's age, occupation, family income, smoking status, alcohol use, antibiotic use, BMI, and weight gain during pregnancy, were collected using standardized questionnaires (Appendix 2).

3.2.3 Nutritional Assessment

Dietary records of mothers were collected at two months of their infant's age using a 3-day diet record on the regular diet during the lactation. Subjects were asked to provide a diet record of every item they drank and ate with quantities over three consecutive days during the one week of the breast milk sample collection date at infants' 2 month of age, place of consumption, types, quantities, and cooking method of the food and beverage consumed (Appendix 4). A face-to-face interview was also conducted to ensure the accuracy of the data reported. Our staff double-checked each item they wrote down and provided the food photographs to them to facilitate the accuracy of the portion size they consumed and reported. The interview was also conducted to ensure no record was missing. The dietary information from the 3-day diet record was entered into ESHA Food Processor to analyze the average daily energy intake, macronutrients, and micronutrients.

3.3 Analysis of Infant Gut Microbiota and Human Milk Oligosaccharide in Breast Milk

3.3.1 Recruitment of Subjects

Mothers and their infants were recruited. Mothers should fulfill the following inclusion criteria: Hong Kong residents having resided in Hong Kong for a continuous period of not less than 18 months; Had normal pre-pregnant BMI (i.e,. 18.5-22.9) and weight gain during pregnancy (i.e., 11-16.4kg); Deliver at full term (>37 gestation weeks); Give birth to singleton infant within the normal birth weight of > 2500g. They have not participated concurrently in any clinical trial or study, Have not taken antibiotics for at least one month before sample collection, Have not used contraceptive medication after giving birth. With no complicated pregnancy such as preeclampsia and gestational diabetes; No special dietary restrictions, for examples gluten-free diets, vegan or any

restrictions due to food allergies; No renal, liver, or thyroid dysfunction, cognitive impairment, or any other indication of a major medical or psychological illness, as judged by the investigators as ineligible to participate the study. The infants should fulfil the following inclusion criteria: full-term and virginal-delivered (>37 gestation weeks), singleton infant with a normal birth weight of >2500g, no known abnormality.

Explanation of the study and informed consent form was given to eligible mothers; they signed the consent form after they received the information about the study (Appendix and Appendix.5). Ethics approval was obtained from the Human Subjects Ethics Sub-Committee (reference number: HSEARS20161230005 and HSEARS20180123009-03), and the biological safety and chemical safety of the study were approved by the Health, Safety and Environment Office, The Hong Kong Polytechnic University, Hong Kong (PolyU).

3.3.2 Fecal Samples and DNA Extraction

Infant fecal samples were collected by mothers at 2, 4, 6, 8,10, and 12 months of age following the instructions from PolyU staff, kept at 4°C, and delivered to the lab within 1 hour after sample collection. The samples were further stored at -80°C until DNA extraction. Genomic DNA was extracted from the fecal samples using the TIANamp Stool DNA Kit (TIANGEN) and stored at -20oC until processed.

3.3.3 Determination of Microbiota: 16S rRNA amplicon sequencing

*The V3-V4 hypervariable region of the b*acteria 16S ribosomal RNA gene was amplified by PCR using primers where a barcode is an eight-base sequence unique to each sample. The amplicon was further purified from the agarose electrophoresis gel using QIAquick Gel Extraction kit (Qiagen), subjected to Qubit Fluorometric quantitation (Thermo

Fisher) and NanoDrop (Thermo Fisher) to check quantity and quality, respectively, and sent to be sequenced by Illumina HiSeq sequencing.

Raw sequence files were demultiplexed, quality-filtered using QIIME2 and Mothur with the following criteria: (i) The 300 bp reads were truncated at any site receiving an average quality score <20 over a 50 bp sliding window, discarding the truncated reads that are shorter than 50bp. (ii) exact barcode matching, 2 nucleotide mismatch in primer matching, and reads containing ambiguous characters were removed. (iii) Only sequences that overlapped longer than 10 bp were assembled according to their overlapped sequence. Reads which could not be assembled were discarded.

Operational Taxonomic Units (OTUs) were clustered with a 97% similarity cut-off using UPARSE software, and chimeric sequences were identified and removed using UCHIME. The taxonomy of each 16S rRNA gene sequence was analyzed by RDP Classifier against the silva (SSU115)16S rRNA database using a confidence threshold of 70%.

3.3.4 Breast Milk Sample Collection

Each mother was asked to provide a mature milk sample (30-40ml) at months 2, 4, 6, 8, 10, and 12 of infant's age. The milk sample was collected when mothers visited our university to do the interview on dietary records and gave us the infant fecal samples. They came to our university either in the morning session or afternoon session. Each mother was allocated a private room, and they were asked to pump out the milk using the sterilized pump and bag provided by our research staff. Before the collection, subjects were asked to clean their nipples and hands using alcohol wipes. The milk was directly collected into the sterile container; the samples were aliquoted into tubes and

stored at -80°C until extraction.

3.3.5 HMO Samples and Standards

Milk oligosaccharide standards, 2'-fucosyllactose (2'FL), lacto-N-neotetraose (LNnT), 3'-sialyllactose (3'SL), and 6'-sialyllactose (6'SL) were purchased from Glycom (Denmark) to build the standard calibration curve. Milk oligosaccharide reference materials with relatively low purities, 3-fucosyllactose (3FL), difucosyllactose (DFL), lacto-N-neohexaose (LNnH), lacto-N-fucopentase I (LNFP I), LNFP II, LNFP III, 3-fucosyl-3'-sialyllactose (FSL), sialyllacto-N-tetraose a (LSTa), LSTc and disialyllacto-N-tetraose (DSLNT) were also provided by Glycom (Denmark) for qualitative analysis.

3.3.6 Breast Milk Sample Preparation

Human milk oligosaccharides (HMO) were extracted from milk samples and reduced using the method described previously (Hong et al., 2014; Wu et al., 2017; Wu et al., 2011) with slight modification. Briefly, 3 mL of raw milk was defatted by centrifuge at 6000 rpm for 30 min. The aqueous layer was collected, mixed with 4 volumes of Folch solution (2:1 chloroform-methanol, v/v), and centrifuged at 6000 rpm for 30 min. The upper layer was collected and deproteinized by ethanol precipitation at a volume ratio of 1:2. The sample mixtures were dried to completion on low heat using a rotary evaporator. The dried samples were dissolved in 3 mL distilled water, and 0.5 mL of the sample solutions were reduced by 0.5 mL of 1 M $NaBH_4$ at 65 °C for 1.5 h, followed by solid phase extraction (SPE) using graphitized carbon cartridges. The SPE cartridges were first washed and activated by 6 mL MilliQ water, 6 mL 80% acetonitrile (ACN) with 0.1% trifluoroacetic acid (TFA, v/v), and then 6 mL MiliQ water. After samples were loaded on SPE cartridges, 30 ml of MilliQ water was used to wash the salts out. HMO were eluted by 6 mL of 20% ACN (v/v) and 6 mL of 40% ACN in 0.05% TFA

(v/v), followed by lyophilization. The samples were reconstituted in 0.5 mL of 0.05 M NaCl solution and diluted 40-fold before mass spectrometry (MS) analysis. Oligosaccharide standards were also reduced by $NaBH_4$, eluted by SPE, and diluted by NaCl solution to plot the calibration curve. The matrix effect was evaluated via standard addition for one of the milk samples by adding a series of HMO standards with different concentrations into the pooled milk sample.

3.3.7 Liquid Chromatography-Mass Spectrometry (LC-MS) analysis

The extracted HMO samples were analyzed using an Agilent 6460 electrospray ionization (ESI) triple quadrupole mass spectrometer equipped with Agilent 1290 liquid chromatography system. A porous graphitic carbon column (100 × 2.1 mm, 3 μm, Hypercarb, Thermo) connected with a guard column (10 × 2.1 mm, 3 μm, Hypercarb, Thermo) was applied to elute different HMOs (Hong et al., 2014). Column temperature was 40 °C. 10 mM ammonium in 0.1% ammonia and 0.1% ammonia (v/v) in ACN were used as solvent A and B, respectively. LC gradient was 60 min: 0-3 min, 1% B; 3-4 min, 1-5% B; 4-20 min, 5-17% B; 20-30 min, 17-42% B; 30-35 min, 42-90% B; 35-50 min, 90%; 50-51 min, 90-1% B; 51-60 min, 1% B. Flow rate increased from 0.1 to 0.15 mL/min in the first 3 min, kept stable at 0.15 ml/min in 3-50 min, decreased to 0.1 mL/min in 50-51min and balanced at 0.1 mL/min in the last 9 min.

MS analysis was operated in positive mode. Gas temperature and sheath gas temperature were 300 °C. Gas flow and sheath gas flow were 11 L/min and 7 L/min, respectively. Nebulizer pressure was 25 psi, and capillary voltage was 4000 V. Fragmentor voltage and collision energy were optimized based on different HMO standards.

3.4 Data Processing and Analysis

Descriptive statistics of both characteristics of mothers and infants were generated and analyzed using the software Statistical Package for Social Sciences (SPSS), version 29.0 (Chicago, USA). The differences in data were analyzed by t-test or ANOVA for continuous outcomes while the chi-squared test of independence for categorical outcomes. $p < 0.05$ was reported as a significant difference. Pearson correlation and Spearman correlation tests to check for the association between the microbiota in stool, HMO in breast milk and maternal nutrient intake. $p < 0.05$ was reported as a significant difference or as trend.

Chapter 4 Maternal Diet during Pregnancy and Lactation in Hong Kong

4.1 Introduction

Maternal nutritional status during pregnancy and lactation has long been recognized as an important factor affecting fetal programming which may increase the risk of long-term diseases in the next generation (Barker, 1997). Recently it is also suggested that maternal nutrition can influence infant gut microbiota development (Sindi et al., 2021).

Relative high fish and seafood consumption but inadequate vegetable and fruit consumption are some diet characteristics in Hong Kong. Seafood and fish are one of the popular choices in Hong Kong's diet. The consumption was 71.8kg per capita, ranking the second in Asia (WWF, 2020). The consumption level was much higher than in the global which the global per capita consumption of fish was estimated at 20.3 kg in 2017 (FAO, 2021). However, the dietary habit is generally considered as unhealthy due to low vegetable and fruit consumption. According to the Report of Health Behaviour Survey 2018/19 published by Centre of Health Protection (CHP), 95.6% of people aged 15 or above did not have adequate daily intake of fruit and vegetables when considering the WHO recommendation which the daily fruit and vegetable consumption should be at least 5 servings per day (CHP, 2020).

To our knowledge, only a few studies on pregnancy diet or lactating diet in Hong Kong could be found so the information reported are limited. One of them is related to inadequate iodine intake during pregnancy in Hong Kong (Tam et al., 2017) in which the dietary intake of iodine of 146 pregnant women was assessed. The study reported

that the median daily intake was 69.5 μg and 83.6% of the mothers had an intake below the 250 μg, the level recommended by the World Health Organization. Another research study on early pregnancy diet in HK was published recently by Tsoi et al. (2022). The average energy intake was 1938kcal in early pregnancy and they found out that 99% of the subjects had excessive sodium intake and only 2.6% could meet the recommended fiber intake. Concerning lactation diet in Hong Kong, our studies (Lu et al., 2022; Wong et al., 2019) reported that mothers had low vegetables and fruit consumption but high seafood consumption (an average of 10 servings per week) during lactation.

The growth of fetus is obvious and significant in the third trimester in addition infant grows fast during early lactation period; the nutritional status of mothers in these two stages is very important. It is worth investigating the maternal diet situation in Hong Kong since the information on maternal diet during pregnancy and lactation in Hong Kong was scarce. Thus, the aim of this study is to assess the maternal diet during the last trimester of pregnancy and lactation.

4.2 Methodology

4.2.1 Subject Recruitment

Subject recruitment took place in health centres in both public and private health sectors, and platforms provided by organizations which promote breast feeding including MaMa Milk Baby Alliance, Leche League and Hong Kong Breastfeeding Mothers' Association. Details refer to Chapter 3.1 and 3.2.

4.2.2 Nutritional Assessment

Information of pregnancy diet were collected using a Food Frequency Questionnaire (FFQ) on the regular diet during the pregnancy while maternal dietary record during lactation were collected at 2 months of their infant's age using a 3-day diet record on the regular diet during the lactation. Mothers were asked to fill in the record and interview was conducted to confirm the input on the day when they visited The Hong Kong Polytechnic University (PolyU). Details refer to Chapter 3.1 and 3.2

4.2.3 Data Processing and Analysis

Descriptive statistics of both characteristics of mothers were generated and analyzed using the software Statistical Package for Social Sciences (SPSS), version 29.0 (Chicago, USA).

4.3 Result

4.3.1 Maternal Diet during Pregnancy

4.3.1.1 Demographic Data of Mothers

A total of 52 women were included in the analysis who had completed the FFQ. The average age of the recruited women was 31.04±0.52 years old, and the mean BMI before pregnancy was 21.04±0.26 kg/m2. The education level of women who had university degree and above was 44.23%. The demographic data of mother are shown in Table 4.1.

4.3.1.2 Daily Dietary Intake during Pregnancy

The daily average energy intake in the study population was 1357.2 ± 80.1 kcal. The average protein, carbohydrates, and fat intake contributed to 23.5%, 54.9%, and 21.9% of energy respectively. The total fiber intake was 14.8 ± 1.0 g. According to the categorization for the food group of MyPlate, the intake of grain, vegetables, fruits, dairy, and protein group were 4.6 ± 0.3 oz, 1.5 ± 0.2 cup, 1.2 ± 0.1 cup, 0.4 ± 0.1 cup and 6.1 ± 0.5 oz respectively. The detail of the average daily intake is shown in Table 4.2.

Table 4. 1 Clinical information of women (n = 52)

Maternal characteristic		
Age (years)	31.04 ± 0.52	
Pre-gestational BMI (Kg/m2)	21.04 ± 0.26	
Weight gain during pregnancy (kg)	13.49 ± 0.66	
Gestational age (weeks)	39.11 ± 0.16	
	n	%
Education background		
Low	28	53.8
High*	24	46.2

Results are shown as mean ± SEM

Table 4.2 Average daily dietary intake of women during pregnancy

Total Kcal	1357.2	±	80.1
Macronutrients			
Protein (g)	82.4	±	6.2
Carbohydrates (g)	180.7	±	9.4
Sugar (g)	39.3	±	2.4
Fructose (g)	7.3	±	0.6
Galactose (g)	0.1	±	0.1
Glucose (g)	5.2	±	0.5
Lactose (g)	2.8	±	0.5
Total Fat (g)	34.0	±	2.6
Saturated fat (g)	9.2	±	0.7
Monounsaturated fat (g)	10.0	±	0.8
Polyunsaturated fat (g)	8.1	±	0.9
Trans fat (g)	0.2	±	0.1
EPA (mg)	132.4	±	72.0
DHA (mg)	187.7	±	72.4
Omega3 (g)	0.7	±	0.1
Omgea6 (g)	3.8	±	0.3
Cholesterol (mg)	302.1	±	21.8
Total dietary fiber (g)	14.8	±	1.0

Table 4.2 Continued

Vitamins			
Vitamin A (RAE;µg)	549.2	±	53.2
Vitamin B1(mg)	1.0	±	0.1
Vitamin B2 (mg)	1.2	±	0.1
Vitamin B3 (NE; mg)	27.5	±	2.1
Vitamin B6 (mg)	1.5	±	0.1
Folate (µg)	231.0	±	17.3
Vitamin B12 (µg)	2.9	±	0.3
Vitamin C (mg)	145.7	±	12.8
Vitamin K (µg)	324.4	±	32.6
Minerals			
Calcium (mg)	581.0	±	38.6
Sodium (mg)	695.3	±	47.7
Potassium (mg)	2314.2	±	143.0
Iron (mg)	12.9	±	0.9
Zinc (mg)	8.1	±	0.6
Iodine (µg)	199.2	±	51.1
Food groups			
Grain (oz)	4.6	±	0.3
Vegetable (cup)	1.5	±	0.2
Fruit (cup)	1.2	±	0.1
Dairy (cup)	0.4	±	0.1
Protein group (oz)	6.1	±	0.5

Results are shown as mean ± SEM.

4.3.2 Maternal Diet during Lactation

4.3.2.1Demographic data of mothers

A total of 38 women were included in the analysis who had completed the 3-day diet record. The average age of recruited mothers was 31.58 ± 0.54 years old and the mean BMI before pregnancy was 20.60 ± 0.32 kg/m2. The education level of women who had university degree and above was 45.0%. The demographic data of mother are shown in Table 4.3.

4.3.2.2 Daily Dietary Intake during Lactation

The recruited mothers were categorized into 3 groups according to the feeding mode at month 2: exclusively breastfeeding (BF mum), mix fed their infant (MF mum), and exclusively fed infants with formula (IF mum). The average energy intake of BF mum and MF mum was more than 2000 kcal. The dietary fiber intake ranged from 8.3 ± 1.9 g in IF mum to 14.8 ± 2.8 g in MF mum. Details of dietary intake during lactation are in Table 4.4

Table 4.3 Clinical information of mothers (n = 38)

Maternal characteristic		
Age (years)	31.58 ± 0.54	
Pre-gestational BMI (Kg/m2)	20.60 ± 0.32	
Weight gain during pregnancy (kg)	13.47 ± 0.64	
Gestational age (weeks)	39.13 ± 0.17	
	n	%
Education background		
Low	20	52.6
High*	18	47.4

Results are shown as mean ± SEM

* High: tertiary education or above

Table 4.4 Average daily dietary intake of mothers during lactation

	Average (n = 38)			**BF mum** (n = 15)			**MF mum** (n = 18)			**IF mum** (n = 5)		
Total Kcal	2170.5	±	95.1	2341.2	±	142.0	2111.6	±	136.3	1870.6	±	302.8
Macronutrients												
Protein (g)	107.7	±	5.2	118.2	±	9.6	106.9	±	5.1	79.2	±	14.3
Carbohydrates (g)	231.3	±	13.9	254.6	±	20.8	214.4	±	21.7	221.9	±	33.5
Sugar (g)	44.7	±	4.9	47.9	±	8.9	41.5	±	6.8	46.5	±	12.2
Fructose (g)	5.1	±	1.4	8.2	±	3.1	3.5	±	1.0	1.5	±	1.0
Galactose (g)	0.2	±	0.1	0.4	±	0.2	0.1	±	0.1	0.0	±	0.0
Glucose (g)	4.7	±	1.2	7.2	±	2.7	3.5	±	1.0	1.4	±	0.7
Lactose (g)	1.5	±	0.6	0.7	±	0.4	2.5	±	1.1	0.2	±	0.1
Total Fat (g)	88.3	±	4.2	91.4	±	7.4	90.1	±	4.9	72.6	±	14.8
Saturated fat (g)	22.0	±	1.2	22.6	±	2.0	22.3	±	1.7	18.9	±	2.6
Monounsaturated fat (g)	28.1	±	1.5	29.5	±	3.0	28.2	±	1.5	23.7	±	4.2
Polyunsaturated fat (g)	16.4	±	1.1	16.8	±	1.5	16.7	±	1.8	14.5	±	4.1
Trans fat (g)	0.4	±	0.5	0.4	±	0.1	0.3	±	0.1	0.4	±	0.1
EPA (mg)	191.8	±	40.8	275.0	±	84.0	160.6	±	45.1	54.0	±	28.2
DHA (mg)	452.6	±	91.8	550.0	±	161.0	445.6	±	137.2	178.0	±	81.3
Omega3 (g)	2.1	±	0.6	1.7	±	0.3	2.8	±	1.1	0.9	±	0.2
Omgea6 (g)	10.1	±	0.7	12.0	±	1.2	9.3	±	0.7	7.2	±	2.0
Cholesterol (mg)	500.8	±	39.9	506.8	±	47.9	538.0	±	66.0	349.1	±	98.4
Total dietary fibre (g)	12.9	±	1.4	12.8	±	0.9	14.2	±	2.8	8.3	±	1.9

Table 4.4 continued

	Average (n = 38)	**BF mum** (n = 15)	**MF mum** (n = 18)	**IF mum** (n = 5)

Vitamins												
Vitamin A (RAE; µg)	321.8	±	63.6	303.2	±	73.1	375.9	±	119.4	182.8	±	49.3
Vitamin B1(mg)	1.2	±	0.1	1.3	±	0.1	1.3	±	0.1	0.9	±	0.1
Vitamin B2 (mg)	1.5	±	0.1	1.5	±	0.1	1.5	±	0.1	1.2	±	0.2
Vitamin B3 (NE; mg)	34.1	±	2.4	40.0	±	4.7	32.0	±	2.3	24	±	5.8
Vitamin B6 (mg)	1.5	±	0.1	1.6	±	0.1	1.5	±	0.1	1.2	±	0.2
Folate (µg)	158.1	±	8.5	169.7	±	13.9	159.7	±	11.1	117.1	±	25.6
Vitamin B12(µg)	5.4	±	1.1	5.9	±	1.6	5.9	±	1.7	2.2	±	0.5
Vitamin C (mg)	58.1	±	6.7	60.9	±	11.7	62.8	±	9.8	32.8	±	7.9
Vitamin K (µg)	65.9	±	10.4	60.5	±	10.9	74.9	±	19.2	49.5	±	23.7
Minerals												
Calcium (mg)	619.5	±	59.8	643.1	±	116.4	641.9	±	79.5	468.4	±	75.9
Sodium (mg)	4011.7	±	188.5	4222.4	±	280.8	4031.0	±	297.0	3310.2	±	403.5
Potassium (mg)	2152.5	±	114.5	2318.9	±	164.1	2062.1	±	126.5	1978.9	±	598.0
Iron (mg)	12.7	±	0.7	13.7	±	1.1	12.7	±	1.1	9.9	±	2.4
Zinc (mg)	10.8	±	0.8	12.1	±	1.8	10.4	±	0.8	8.7	±	1.7
Iodine (µg)	47.2	±	6.7	29.6	±	5.6	51.4	±	9.6	85.0	±	28.7
Food Groups												
Grain (oz)	6.1	±	0.6	7.4	±	0.8	5.5	±	0.9	4.3	±	0.5
Vegetable (cup)	0.5	±	0.1	0.6	±	0.1	0.5	±	0.1	0.3	±	0.1
Fruit (cup)	0.5	±	0.1	0.7	±	0.2	0.4	±	0.1	0.2	±	0.2
Dairy (cup)	0.2	±	0.0	0.1	±	0.0	0.2	±	0.1	0.5	±	0.2
Protein group (oz)	8.9	±	0.7	9.1	±	1.5	9.6	±	0.7	5.6	±	1.3

4.4 Discussion

4.4.1 Energy Intake during Pregnancy

Pregnant women need the energy to support their everyday needs and extra energy to support the development of the maternal tissues and fetal growth and development (IOM, 2009). In Hong Kong, the recommendation of energy requirement follows a Joint FAQ/WHO/UNU Expert Consultation in 2011, which is 475 kcal/d extra in the third trimester (DH, 2022); while in US, the Dietary Guideline for Americans 2020 - 2025 recommends the pregnant women in third trimester to consume 452kcal/d more (OASH, 2022). The energy requirement for moderately active, non-pregnant women is approximately 1,600 to 2,400 kcal per day (CDC, 2022). The estimated energy requirement of pregnant women recommended by Chinese DRI is 2250 - 2850kcal per day.

The dietary information during pregnancy in our study was collected through FFQ in which the subject need to answer a questionnaire that includes a list of foods by stating out the item that they ate with frequency and quantity within a period of time which was 90 days in our study. The result indicated that the average energy intake was only 1357.2 ± 80.1 kcal, which was lower than the studies conducted in southwest China which collected the dietary information through FFQ and 24-hour recall and Japan which collected the dietary data through a 3-day food dietary record. The energy intake was 2065kcal (Zhang et al., 2020) and 1680kcal (Imai et al., 2021) respectively.

The energy intake was very low when compared to those reported by other studies and the recommended energy requirement. More than 90% of the subjects had energy intake less than 2250 kcal a day. However, it may not reflect the real energy intake because all

mothers had normal gestational weight which was in average 13.47 kg. If energy intake is inadequate, the fetal development will be affected, and will be reflected in the gestational weight gain.

4.4.2 Macronutrient Intake during Pregnancy

Macronutrients include carbohydrates, proteins and fats. In addition to energy supply, protein and fat are important structural components for fetal development. According to Institute of Medicine (IOM), the US Recommended Dietary Allowance (RDA) for carbohydrate during pregnancy is 175g/d and 71g/d for protein (IOM, 2009). While according to Chinses Nutrition Society (CNS), the Chinese Dietary Reference Intake (DRI) 2013, Estimated Average Requirement (EAR) for carbohydrate is 130g/d and RNI for protein is 85g/d (CNS, 2013).

EAR is defined by the criterion of adequacy to meet the requirement of half of the apparently healthy individuals in a particular life stage. It is also used to set up RDA which is recommended intake level for individuals and is adequate to fulfil the requirement of 97-98% of the apparently healthy individuals in a particular life stage. When individuals follow the recommendation, the risk of developing negative function outcome of a particular nutrient will be reduced (Trumbo et al., 2001).

Figure 4.1 shows the box and whisker plot of carbohydrate and protein intake during pregnancy and the recommended level of US and Chinese DRI. The average protein intake was 82.4g while the carbohydrate intake was 180.7g. Regarding the carbohydrate intake, 71% of subjects could reach 130g/d while only 46% of subject could reach 175g/d. 52% of subjects did not reach the recommendation of RDA for protein (USDA)

while 61.5% did not reach the recommendation of Recommended Nutrient Intake (RNI) for protein (Chinese DRI 2013).

According to USDA Acceptable Macronutrient Distribution Range (AMDR), % of energy from proteins, carbohydrates, and fats should be 10-35%, 45-65%, and 20-35%, respectively, while Chinese DRI recommended 50 – 65% of energy from carbohydrates while 20 – 30 % from fat. AMDR is a recommended range of intake for energy source which is associated with a lower risk of chronic disease when intakes of essential nutrients are adequate. Therefore, when an individual has consumption over the AMDR, the risk of developing chronic diseases may be higher (Trumbo et al., 2002). In our result, the average protein, carbohydrates, and fat intake contributed to 23.5%, 54.9%, and 21.9% energy respectively, meeting the recommendation of AMDR. Considering the fat intake, 36.5% of the subjects had less than 20% energy from fat which was less than the AMDR suggested by Chinese DRI while 5.8% had more than 30% from fat in the diet. However, the total intake of carbohydrate and fat may not be adequate for energy supply. The average fat intake during pregnancy in our study was only 34g which could only supply roughly 300kcal in a day. The reason may be due to underestimation of intake using FFQ as a reporting tool.

4.4.3 Dietary Fiber Intake during Pregnancy

Dietary fiber is important for bowel movement, removing bile from our body and stabilizing blood glucose. According to the Centre for Health Protection, Department of Health, the dietary fiber intake should not less than 25g per day (CHP, 2017) while the US Adequate Intake (AI) for dietary fiber for pregnant women is 28g. AI for dietary fiber (14g / 1000kcal) is based on the observed level which can protect against coronary

heart disease (Oria & Kumanyika, 2017). From our data, the average dietary fiber intake was only 12.8g, which was just around 50% of the recommended level. Only 5 out of 52 subjects had more than 25 g dietary fiber a day (Figure 4.2). Since the reported energy intake was low which was only around 1400kcal in our study group, the recommended intake level of dietary fibre was adjusted based on the energy intake which is 19g (14g / 1000 kcal). Still only 12 out of 52 subjects had met the recommended intake level. The inadequate intake was comparable to the study in Hong Kong (Tsoi et al., 2022) and Japan (Imai et al., 2021) which both studied the intake during the early pregnancy. The intake in our group was a bit lower than the average intake of pregnant women during third trimester in China which was 15.8g (Liu et al., 2015). The low intake may be due to low consumption of whole grain products, vegetables, and fruits, or partly due to underestimation of food intake using FFQ.

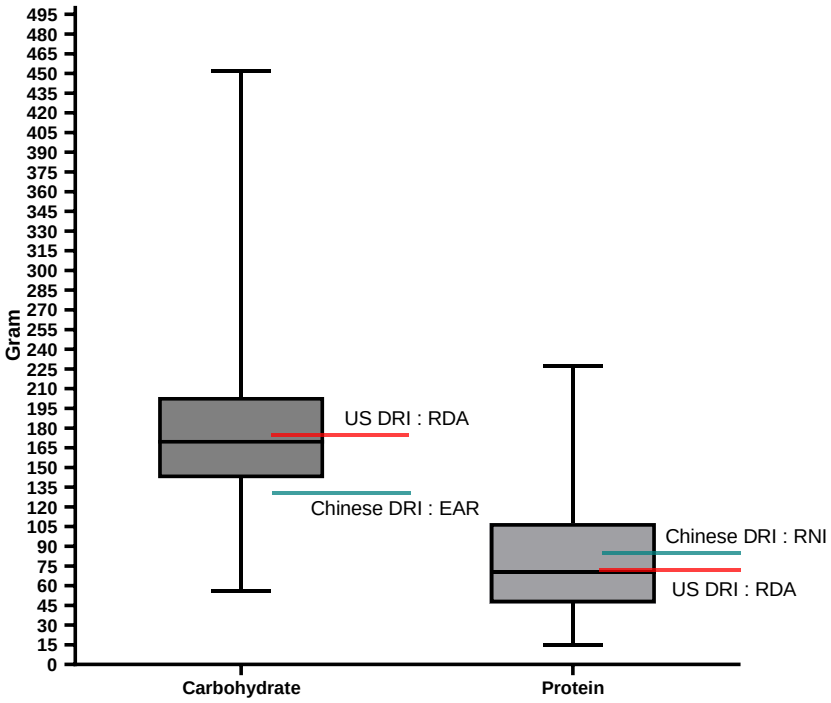


Figure 4. 1 Box and Whisker plot of carbohydrate and protein during pregnancy.
DRI: Dietary Reference Intake; RDA: Recommended Dietary Allowance; EAR: Estimated Average Requirement; RNI : Recommended Nutrient Intake

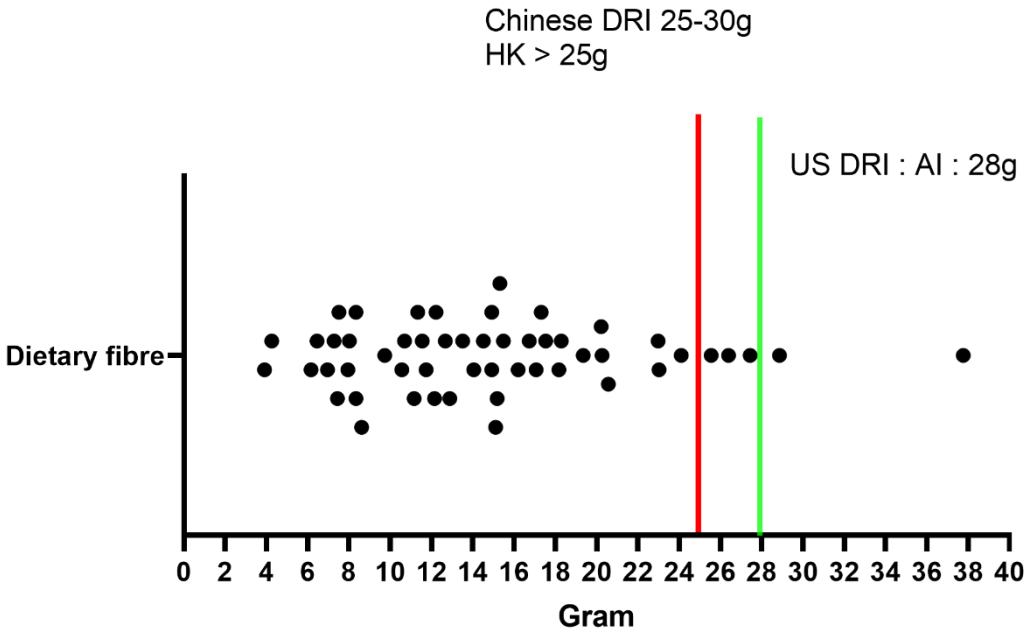


Figure 4. 2 Individual plot of dietary fiber intake during pregnancy.
DRI: Dietary Reference Intake; AI : Adequate Intake

4.4.4 Micronutrient Intake during Pregnancy

Micronutrient includes vitamins and minerals are essential for both mother own use and fetal development. Pregnant women like all other individuals need all vitamins and minerals but there are some special considerations during pregnancy. For example, vitamin Bs especially folate is important for neural tube development. Iron is important for blood and cell development while iodine is important for fetal neurocognitive development. Table 4.5 shows the recommended level of some vitamins and mineral set by Chinese DRI and US DRI and the mean intake level in our study. Most of the vitamins and minerals intake were inadequate. More than 70% of subject did not reach the recommendation of Chinese RNI / USA RDA on intake of Vitamin A, Vitamin B1, B2 and B6 intake while minerals include calcium, iron and iodine. According to EAR cut point method which is a way to assess the nutrient adequacy in a group (IOM, 2000), there was still 70% or more of the subject did not met the recommendation on the intake of Vitamin B1, calcium, iron and iodine. The inadequacy may affect the visual, neural and bone development of the fetus. However, most of the subjects in our study consumed more than 1 types of supplements during pregnancy.

Table 4. 5 The recommended level of some vitamins and mineral set by Chinese DRI and US DRI and intake level in our study (n=52).

	Average intake	Percentile			Chinese DRI RNI/AI	% of inadequacy	Chinese DRI EAR	% of inadequacy	US DRI RDA/AI	% of inadequacy	US DRI EAR	% of inadequacy
		25^{th}	50^{th}	75^{th}								
Vitamin A (μg RAE)	549.2	282.6	448.1	776.6	750	75.0	530	61.5	770	75.0	550	61.5
Vitamin B1(mg)	1.0	0.5	0.8	1.2	1.5	88.5	1.2	76.9	1.4	86.5	1.2	76.9
Vitamin B2(mg)	1.2	0.7	1.1	1.5	1.5	76.9	1.2	53.8	1.4	69.2	1.2	53.8
Vitamin B3(mg)	27.5	16.2	24.4	33.2	12	9.6	10	3.8	18	28.8	14	17.3
Vitamin B6(mg)	1.5	0.8	1.4	2.0	2.2	80.8	1.9	73.1	1.9	73.1	1.6	63.5
Folate (μg)	231.0	133.0	228.4	285.8	600	100.0	520	94.2	200*	42.3	520	94.2
Vitamin B12 (μg)	2.9	1.6	2.5	4.0	2.9	63.5	2.4	48.1	2.6	51.9	2.2	48.1
Vitamin C (mg)	145.7	82.3	116.9	186.1	115	48.1	95	40.4	85	32.7	70	15.4
Vitamin K (μg)	324.4	159.2	253.0	410.5	80	13.5	--	--	90	13.5	--	--
Calcium (mg)	581.0	392.1	577.9	699.6	1000	96.2	810	80.1	1000	96.2	800	78.8
Sodium (mg)	695.3	424.4	622.7	858.6	1500	NA	--	--	1500	NA	--	--
Potassium (mg)	2314.2	1401.3	2213.8	3040.7	2000	40.4	--	--	2900	71.2	--	--
Iron (mg)	12.9	8.0	11.2	16.7	29	96.2	22	94.2	27	96.2	22	94.2
Zinc(mg)	8.1	5.2	7.8	11.1	9.5	71.1	7.8	50.0	11	75.0	9.5	71.1
Iodine (μg)	199.2	34.0	63.6	105.0	230	82.7	160	80.8	220	80.8	209	80.8

*Assume 400μg will be taken from supplement

4.4.5 Food Group Intake during Pregnancy

Table 4.6 shows the comparison between the intake in our study with the recommended intake from Hong Kong, China and US. Regarding the food group intake, almost 90% were lower than the recommendation issued by China, US and Department of Health, Hong Kong except the intake of protein group which was only 69.2% inadequacy. Therefore, this is the reason why more than 90% of the subjects did not reach the energy recommendation. When adjusting the food group intake's recommendation based on their energy intake level (~1400-1600 kcal), more than 50% of the individuals in the groups did not have enough grains, vegetables, fruits and dairy intake. The percentage of inadequacy of fruits and dairy intake was 78.8% and 98.1%, respectively.

4.4.6 Overall Pregnancy Diet in Hong Kong

In general, the intake during pregnancy was inadequate across all the food group. The problem of inadequate dairy production consumption was serious, 92.3% had less than 1 cup of dairy product per day leading to inadequate calcium intake.

In addition to inadequate food intake, one of the reasons for low food consumption may be due to underestimation of intake using FFQ as a reporting tool. Dietary information recorded by the FFQ has one major limitation which is recall bias so that the intake may be recorded wrongly due to errors in memory especially the information collected in this study required women to report the diet according to their intake in the past 90 days. The low fat intake may also possibly be due to oil and sauce intake underestimation since the information on cooking method was missing when the collecting dietary data. Despite the limitation, our study demonstrated the protein intake during pregnancy in Hong Kong was at suitable level even under this underestimated situation.

Table 4.6 The food group intake in our study and the recommended intake from Hong Kong, China and US.

Food group	Average	Percentile			HK (DH, 2022)	China#	US (USDA, 2016)	% of inadequacy	Adjustment based on 1400kcal	% of inadequacy	Adjustment based on 1600kcal	% of inadequacy
		25th	50th	75th								
Grains (oz)	4.6	3.2	4.3	5.8	3.5 – 5 servings*	300– 350g	8 ounces	94.2%	5 ounces	69.2%	5 ounces	69.2%
Vegetables (cup)	1.5	0.5	1.4	2.1	4 – 5 servings**	400 – 500g	3 cups	90.4%	1½ cups	53.8%	2 cups	73.1%
Fruits (cup)	1.2	0.6	1.0	1.4	2 – 3 servings***	200 – 350g	2 cups	86.5%	1½ cups	78.8%	1½ cups	78.8%
Meat, fish, egg and alternative /Protein group (oz)	6.1	3.0	5.3	7.6	5 – 7 servings****	175 – 225g	6.5 ounces	69.2%	4 ounces	36.5%	5 ounces	44.2%
Milk and alternatives (cup)	0.4	0.0	0.2	0.5	2 cups	300g – 500g	3 cups	98.1%	3 cups	98.1%	3 cups	98.1%

*1 serving = 2 slices of bread ~ 2 ounces of Grains in US guidelines
** 1 serving = 1 bowl of uncooked vegetable ~ 1 cup in US guidelines
*** 1 serving = 1 apple of lady's fist ~ 1 cup in US guidelines
**** 1 serving = 1 egg/ 30g cooked meat ~ 1 ounce in US guideline
# From Chinese Nutrition Society - http://dg.cnsoc.org/upload/affix/20220819170653935.jpg

4.4.7 Energy Intake during Lactation

Lactating women need extra energy to produce breast milk and support nutrients to the growth of infants. For exclusively breastfeeding, it roughly takes 500kcal to produce human milk for the first 6 months. After 6 months and solid food introduction, it costs around 400kcal/day. Since mothers has stored some energy supply in tissue during pregnancy, mothers only need roughly 330kcal extra from diet in the first 6 months. (Reader & Franz, 2004). According to Centers for Disease Control and Prevention (CDC), the energy requirement is approximately 2,000 to 2,800 kcal per day (CDC, 2022). While 2300kcal to 2900kcal is the estimated energy requirement proposed by Chinese DRI 2013.

3-day diet record was used to analyse the maternal diet during lactation at month 2 of the infant age. The average energy intake of BF mum and MF mum was 2341.2 ± 142.0 kcal and 2111.6 ±136.3kcal respectively. The results were consistent with the energy intake during lactation in Hong Kong reported by Lu et al. (2022) and Wong et al. (2019) which was around 2393.1kcal and 2370.2kcal. The energy intake in Hong Kong was lower than the intake of exclusively breastfed mother in Manila which was 2516.7kcal/day (Angeles-Agdeppa et al., 2022). The reason may be due to some obese mothers were included in the study in Manila. The intake in Hong Kong was a bit higher than the intake in China including both urban and rural area in 2018 which ranged from 1327.45 to 2085.97 kcal (Ding et al., 2020). The average energy intake in our study fell within the energy requirement range provided by CDC and Chinese DRI. In our study, 60.0% of the BF mum had less than 2300kcal intake a day.

4.4.8 Macronutrient Intake during Lactation

In addition to energy supply, carbohydrate and proteins are important elements for producing lactose, protein, antibodies in human milk for infant. The RDA for carbohydrate during lactation is 210g/d and 71g/d for protein while Chinese DRI recommended the EAR for carbohydrate intake is160g/d and RNI for protein is 80g/d.

The average intake of BF mum for carbohydrate and protein was 254.6g ± 20.8g and 118.2g ± 9.6g respectively. Figure 4.3 shows the box and whisker plot of carbohydrate and protein intake during lactation. 93.3% of them met both US RDA and Chinese RNI for protein intake. Regarding the carbohydrate intake, 93.3% of subjects could reach 160g/d while only 80.0% of subject could reach 210g/d. Our result is also comparable to the result reported by Wong et al. (2019) which the average carbohydrate and protein intake was 258.6 and 112.1g respectively.

Regarding the energy distribution, % of energy from proteins, carbohydrates, and fats of BF mum was 20.2%, 43.8%, and 35.0% respectively. The average dietary fat intake of our study was out of the AMDR suggested by the Chinese DRI (20 – 30%). 66.7% of the subjects in BF mum had too much fat intake which contributed to more than 30% of their daily energy intake. The observation was consistent with the finding in Wong et al. (2019) that high protein and fat intake during lactation.

4.4.9 Dietary Fiber Intake during Lactation

The average dietary fiber intake in BF mum and MF mum were 12.8g/d and 14.2g/d respectively which was lower than the recommended level 25g/day suggested by Hong Kong Department of Health, or AI from Chinese DRI (25 – 30g) or US DRI(29g/day).

The intake level was similar to the intake of breastfeeding mum in Manila and Japan which was 11g/d (Angeles-Agdeppa et al., 2022) and 11.66g (Higurashi et al., 2023) , respectively. The intake level was also comparable to the Chinese lactating women in Nanjing city which the median intake was 15.0g (Ding et al., 2021). While it Figure 4.4 indicates the individual plot of the BF and MF mum. It is clearly showed that 100% of the BF mum did not reach the recommended level of dietary fiber. It has been proposed that dietary fiber intake can alter maternal gut microbiota and lead to adverse health outcomes (Gershuni et al., 2021). Therefore, more education should be delivered to enhance the maternal intake of dietary fiber. High source of dietary fiber includes whole grain products, lentils, and some vegetables such as broccolis and corns.

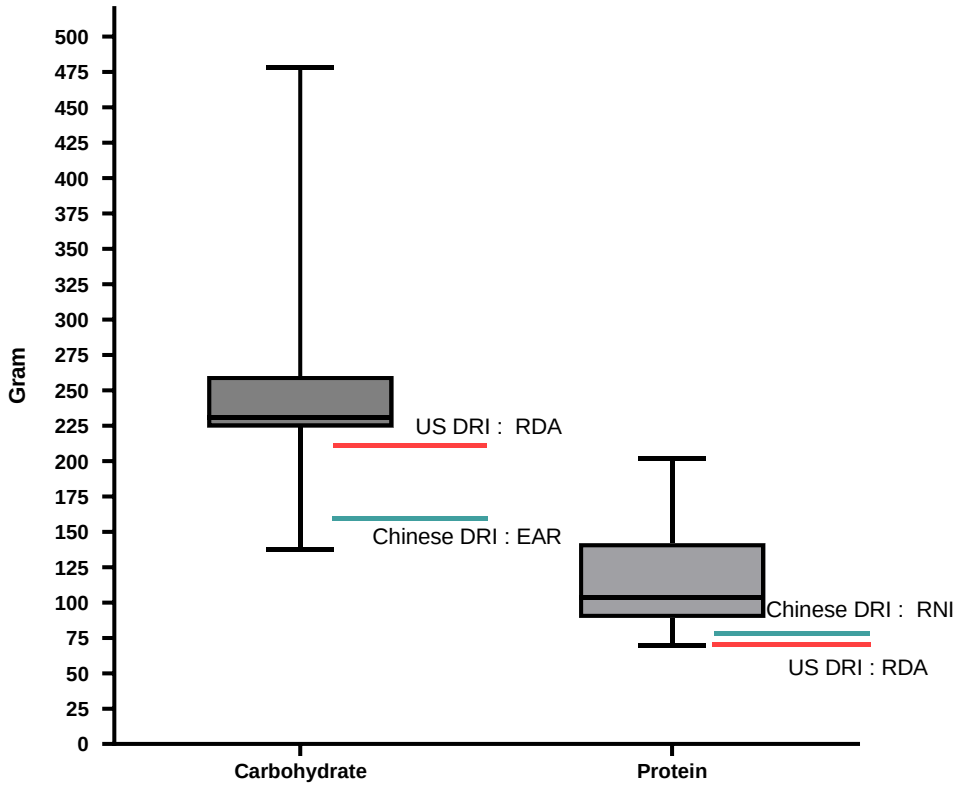


Figure 4. 3 Box and Whisker plot of carbohydrate and protein during lactation.
DRI: Dietary Reference Intake; RDA: Recommended Dietary Allowance; EAR: Estimated Average Requirement; RNI : Recommended Nutrient Intake

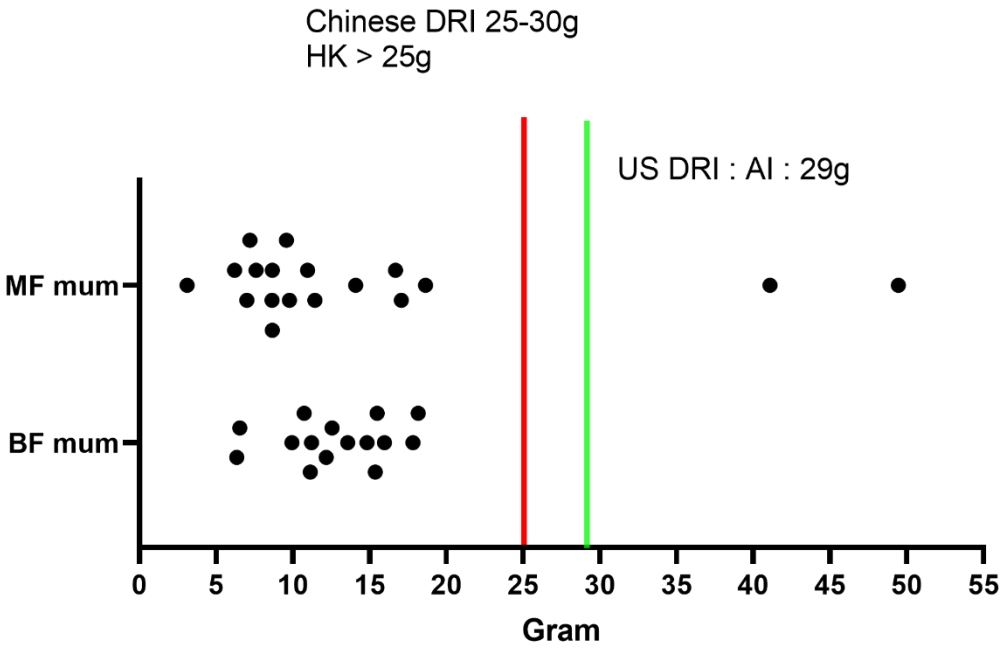


Figure 4.4 Individual plot of dietary fiber intake of BF and MF mum during lactation MF mum: mothers who fed their baby with both breast milk and infant formula at month 2; BF mum : mothers who exclusively breastfed their child at month 2; DRI: Dietary Reference Intake; AI: Adequate Intake

4.4.10 Micronutrient Intake during Lactation

Table 4.7 shows the recommended level of some vitamins and mineral set by Chinese DRI and US DRI and the mean intake level in our study. Most of the vitamin and mineral intake were lower than the recommendation. 100% of BF mum did not reach the RDA/RNI of Vitamin A, Folate and Iodine. Almost 90% of the BF mothers did not achieve the recommendation of Vitamin C and 80% for Calcium. According to the EAR cut point method, more than 80% of the individuals in group was at risk of Vitamin A, Folate, Vitamin C, Zinc and Iodine. The low intake of vitamin A was consistent with the study reported by Lu et al. (2022) which studied the dietary vitamin A intake during lactation in Hong Kong.

Iron recommendation is quite different between Chinese DRI and US RDA, which is 24mg and 9mg, respectively. 100% of the BF mum subject did not achieve the recommendation of Iron suggested by Chinese DRI, but only 13.3% did not get 9mg Iron. Based on the intake information, most of the BF mum were at suboptimal level of micronutrient intake from diet.

Most of the micronutrient intake were inadequate except sodium. There is excess intake of sodium during breastfeeding was observed in our study and it was consistent with the observation of a study which investigated the maternal diet during early pregnancy in Hong Kong (Tsoi et al., 2022). The average intake of sodium in our study was also more than 4000mg in both BF mum and MF mum. The high intake of sodium was consistent also in that study which women consumed around 4000mg sodium in Hong Kong during early pregnancy (Tsoi et al., 2022). High sodium intake should be avoided since overconsumption will increase the risk of cardiovascular diseases.

However, within this study population, only 2 out of 38 mothers did not consume any supplement during lactation. Amongst those who took supplements, in general they took more than 1 types of supplements. 25 mothers took DHA supplement, 23 of them took multivitamin supplement, 15 mothers took folic acid while 13 mothers took calcium supplement. The supplements can complement the inadequacy in diet. There are many brands of multivitamin on the market, more than 40% of the mothers in our study took the same brand containing more than 450µg RAE vitamin A, 70mg vitamin C, 600µg folic acid and 225µg iodine which can meet the lactation requirement except Vitamin A. There is only around 250mg calcium in those multi vitamin and mineral tablet, however some mothers took calcium tablet in extra.

4.4.11 Food Group Intake during Lactation

100% of the BF mum had lower vegetables, fruits and diary intake than the recommendation of Hong Kong, China and US (Table 4.8). There was almost no dairy consumption so that the calcium intake of this group was also inadequate. When vegetable and fruits intake was not enough, the vitamin and mineral intake in general will be inadequate, especially for Vitamin Bs, C and potassium. However, 46.7% of BF mum had more than 8.5oz of protein group which was much higher than the recommendation (6.5oz). This observation was consistent with the characteristics of diet in Hong Kong which the fish and seafood consumption was high while vegetables, fruit and dairy intake was low.

Table 4.7 The recommended level of some vitamins and mineral set by Chinese DRI and US DRI and intake level of BF mum in our study.

	Average	Percentile			Chinese DRI RNI/AI	% of inadequacy	Chinese DRI EAR	% of inadequacy	US DRI RDA/AI	% of inadequacy	US DRI EAR	% of inadequacy
		25th	50th	75th								
Vitamin A (μg RAE)	303.2	119.4	221.7	453.2	1300	100	880	93.3	1300	100	900	93.3
Vitamin B1(mg)	1.3	1.0	1.3	1.5	1.5	66.7	1.2	40.0	1.4	60	1.2	40.0
Vitamin B2(mg)	1.5	1.2	1.5	1.7	1.5	46.7	1.2	20.0	1.6	66.7	1.3	26.7
Vitamin B3(mg)	40.0	26.6	35.7	61.7	15	6.7	12	0	17	6.7	13	0
Vitamin B6(mg)	1.6	1.2	1.5	1.8	1.7	60.0	1.4	40.0	2.0	81.3	1.7	60.0
Folate (μg)	169.7	134.3	169.2	193.4	550	100	450	100	500	100	450	100
Vitamin B12 (μg)	5.9	2.0	3.1	6.4	3.2	53.3	2.6	33.3	2.8	40.0	2.4	33.3
Vitamin C (mg)	60.9	35.4	43.0	74.5	150	93.3	125	93.3	120	93.3	100	93.3
Vitamin K (μg)	60.5	24.1	53.0	99.7	80	66.7	--	--	90	73.3	--	--
Calcium (mg)	643.1	377.9	501.1	659.2	1000	86.7	810	80.0	1000	86.7	800	80.0
Sodium (mg)	4222.4	3689.7	4015.2	4928.7	1500	0	--	--	1500	0	--	--
Potassium (mg)	2318.9	1785.8	2364.6	2665.7	2400	53.3	--	--	2800	86.7	--	--
Iron (mg)	13.7	9.9	14.1	16.6	24	100	25	100	9.0	13.3	6.5	0
Zinc(mg)	12.1	7.6	11.2	12.4	12.0	66.7	9.9	33.3	12.0	66.7	10.4	40.0
Iodine (μg)	29.6	16.3	25.7	30.6	240	100	170	100	290	100	209	100

Table 4.8 The food group intake in our study and the recommended intake from Hong Kong, China and US.

Food group	Average intake	25th percentile	50th percentile	75th percentile	HK (DH, 2022)	China#	US (USDA, 2016)	% of inadequacy
Grains (oz)	7.4	5.0	7.3	9.8	4 – 5 servings*	300 – 350g	8 ounces	53.3
Vegetables (cup)	0.6	0.4	0.5	0.9	4 – 5 servings**	400 – 500g	3 cups	100
Fruits (cup)	0.7	0.1	0.6	1.4	3 servings***	200 – 350g	2 cups	100
Meat, fish, egg and alternative /Protein group (oz)	9.1	4.8	7.2	12.2	6 – 7 servings****	175 – 225g	6.5 ounces	40.0
Milk and alternatives (cup)	0.1	0.0	0.1	0.1	2 cups	300g – 500g	3 cups	100

*1 serving = 2 slices of bread ~ 2 ounces of Grains in US guidelines
** 1 serving = 1 bowl of uncooked vegetable ~ 1 cup in US guidelines
*** 1 serving = 1 apple of lady's fist ~ 1 cup in US guidelines
**** 1 serving = 1 egg/ 30g cooked meat ~ 1 ounce in US guideline
# From Chinese Nutrition Society - http://dg.cnsoc.org/upload/affix/20220819170712081.jpg

4.4.12 Overall Dietary Recommendation for Pregnant and Lactating Women in Hong Kong

To get a well-balanced diet, more whole grain products, vegetables, fruit and dairy products should be consumed in both pregnant women and lactating women so as to increase the dietary fiber, vitamin Bs and C, potassium and calcium intake.

In order to get higher amount of dietary fiber in the diet, replace the refined grain in diet to whole grain products such as whole bread, red/brown rice and whole noodles instead of white bread, white rice and refined noodles. More vegetables and fruits should be included in the diet. High dietary fiber vegetables can be chosen also for example like broccoli, carrot, or corn. Vegetables and fruits not only can supply dietary fiber, it can also supply vitamin A, Bs and vitamin C.

Dairy products should also be included in the diet but the products should be non-fat or low fat to provide calcium content for bone health in mothers especially during lactation. In theory, the calcium in human milk is quite stable and independent of maternal calcium intake (Kent et al., 2009). However, if inadequate calcium intake in mothers, it may cause bone to release calcium for milk production (Bae & Kratzsch, 2018). Since the fat intake during lactation in BF mum was high, low fat cooking methods or nonfat products should be chosen. It has been reported that the DHA and α-linolenic acid intake and their levels in milk were positively associated (Wong et al., 2019), therefore healthy fat should be chosen in the diet.

Most subjects had taken multi-vitamin and mineral supplements during pregnancy and lactation. However whole food and healthy diet are still essential to our life, nutritional supplements should not be regarded as substitutes (Tsoi et al., 2022). It is still important

to understand the dietary intake of both pregnant and lactating women in Hong Kong to lower the risk of over consumption especially most subjects in our study took more than 1 type of supplement and some of them took multivitamin tablet and folic acid supplementation at the same time.

4.4.13 Strength and Limitation of This Study

Regarding the strength of our study, it is one of the few studies to examine the maternal diet during lactation in Hong Kong. 3-day diet record were used to collect the dietary information which can minimize the chance of recall bias and error in memory. A follow up face to face interview were conducted to confirm the intake with the participants could further enhance the accuracy of the lactating diet record. However, there are some limitations in our study, in addition to relative small sample size, FFQ was used to evaluate the diet during pregnancy in HK, the recall bias and error in memory may lead to the underestimation of the intake especially it is well known that the unhealthy food items may be underreporting. The missing information on cooking method in FFQ will also underestimate the oil and sauce consumption and thus the fat intake.

To conclude, the diet analysis of FFQ showed that the average intake of energy, vegetables, fruits, and dairy products in pregnant women was not enough when compared with the recommendation of MyPlate and Department of Health (HK). On the other hand, the protein intake was at suitable level. Regarding the lactating diet, similar result was obtained that the intake of vegetables, fruits, and dairy products was much lower than the recommendation. However, the intake of sodium during lactation was excessive. The result of this study suggested that maternal diet especially during lactation was not healthy.

Chapter 5 Gut Microbiota Development in Hong Kong infants

5.1 Introduction

Gut microbiota has been shown to link with human health. The dysbiosis of gut microbiota will increase the risk of diseases. The first colonization of microorganism in gut occurs at birth and gradually matures and develops until 3 years old (Milani et al., 2017). The infant gut microbiome plays an important role in the development of immune system and against the invasion of pathogens (Olin et al., 2018). Moreover, it has been reported that disruption of early colonization of gut microbiota may increase the risk of asthma, allergy and immune inflammatory response (Gensollen et al., 2016).

Various factors may affect the development of infant gut microbiota such as dietary habit of mother in pregnancy (Garcia-Mantrana et al., 2020) or lactation (Alsharairi, 2020) and infant feeding pattern (Ho et al., 2018). Quite a few studies on the impact of maternal diet during pregnancy on infant gut microbiota have been reported (Babakobi et al., 2020; Garcia-Mantrana et al., 2020; Lundgren et al., 2018). Yet, the effects of maternal diet during lactation were limited (Sindi et al., 2021; Sindi et al., 2022).

Other than the maternal diet, early feeding pattern has also been proposed to be the single most significant factor affecting the infant gut microbiome (Stewart et al., 2018). World Health Organization (WHO) recommends baby should be breastfed exclusively up to 6 months of age and continued breastfeeding along with complementary foods up to two years of age. Breast milk not only contains all the essential nutrients for children to grow, but also antibodies which can protect them from common illness such as diarrhoea (WHO, 2023)

Breast milk or its substitutes are the first source of nutrient for infants who are normally fed with either breast milk or infant formula in the first 4 – 6 months. Breastfeeding, this mode of feeding, in early life is one of the factors modifying the gut microbiota in infants (Serino et al., 2017). The research conducted by Penders et al. (2006) was one of the early studies showing that the infant gut microbiota was different in group with exclusively breastfed and infant formula fed.

Metagenomics analysis, an extensive sequencing to study the composition and function of microbiota, has been carried out on fecal samples of infants in the US and Europe. Since there is not much data in Hong Kong, the aim of this study is to examine the development of Hong Kong infants' gut microbiota during the first year of life and explore if infant gut microbiota is shaped by maternal diet and early feeding practices.

5.2 Methodology

5.2.1 Recruitment of Subjects

Mothers and their infants were recruited. Ethics approval was obtained by Human Subjects Ethics Sub-Committee (reference number: HSEARS20161230005 and HSEARS20180123009-03). Background information including mother's age, occupation, family income, smoking status, alcohol use, antibiotic use, BMI and weight gain during pregnancy as well as infants' birth weight and anthropometric data were collected using standardized questionnaires. Detail refers to Chapter 3.2.

5.2.2 Fecal Samples and DNA Extraction

The fecal samples of infants were collected by mothers at their 2, 4, 6, 8 ,10 and 12

months of age following the instructions from PolyU staff, kept at 4°C and be delivered to the lab within 1 hour after sample collection. The procedure of samples extraction and 16S rRNA sequencing refers to Chapter 3.3.

5.2.3 Nutritional Assessment

Dietary records of mothers were collected at 2 months of their infant's age using a 3-day diet record on the regular diet during the lactation. Detail refers to Chapter 3.2.

5.2.4 Data Processing and Analysis

Data were analyzed by ANOVA or t-test. Pearson correlation and Spearman correlation tests to check for association between the microbiota in stool and maternal nutrient intake. $p < 0.05$ was reported as significant difference or as trends.

5.3 Result

5.3.1 Alpha Diversity of Infant Gut Microbiota

The Shannon index was used to estimate the alpha diversity, which presents the complexity of gut microbiota composition within the same group, of the species in infants' gut during the first year of life. Infants were divided into 3 groups according to the feeding mode: exclusively breastfeeding (BF), mix feeding (MF), and exclusively feeding with formula (IF). The alpha diversity was lower in month 2 of all infants when compared to other months in 3 groups of infants ($p<0.05$) (Figure 5.1).

5.3.2 Effect of Feeding Modes on Alpha Diversity

Considering the Shannon index of different feeding mode regardless the age of the sample collected, it showed that alpha diversity in stool of formula fed baby were higher

than those in breastfed and mix fed, suggesting a more diverse gut microbial community of infants receiving infant formula than those fed with breast milk (Figure 5.2). When referring to the samples of month 2 and month 4, lower faith PD and Shannon index was observed in infant of BF than the IF one however the result did not reach statistical significance (Figure 5.3).

5.3.3 Infant Gut Microbiota at Phylum Level during the First Year of Life

In this study, a total of 173 fecal samples over the first year of life were collected at month 2, 4, 6, 8, 10 and 12 and 158 samples were included in the analysis. The fecal samples collected are categorized based on the feeding practice: Exclusively breastfed (BF), exclusively formula fed (IF) and mix fed (MF). Figure 1 shows the gut microbiota at phylum level during the first year of life. The 3 predominant Phyla were Proteobacteria, Firmicutes and Bacteroidetes within the fecal samples across the first year of life. Proteobacteria level was the highest in month 2 than other months, Bacteroidetes and Firmicutes were dominant after month 4. The gut microbiota during the first year was highly dynamic regardless the feeding modes (Figure 5.4).

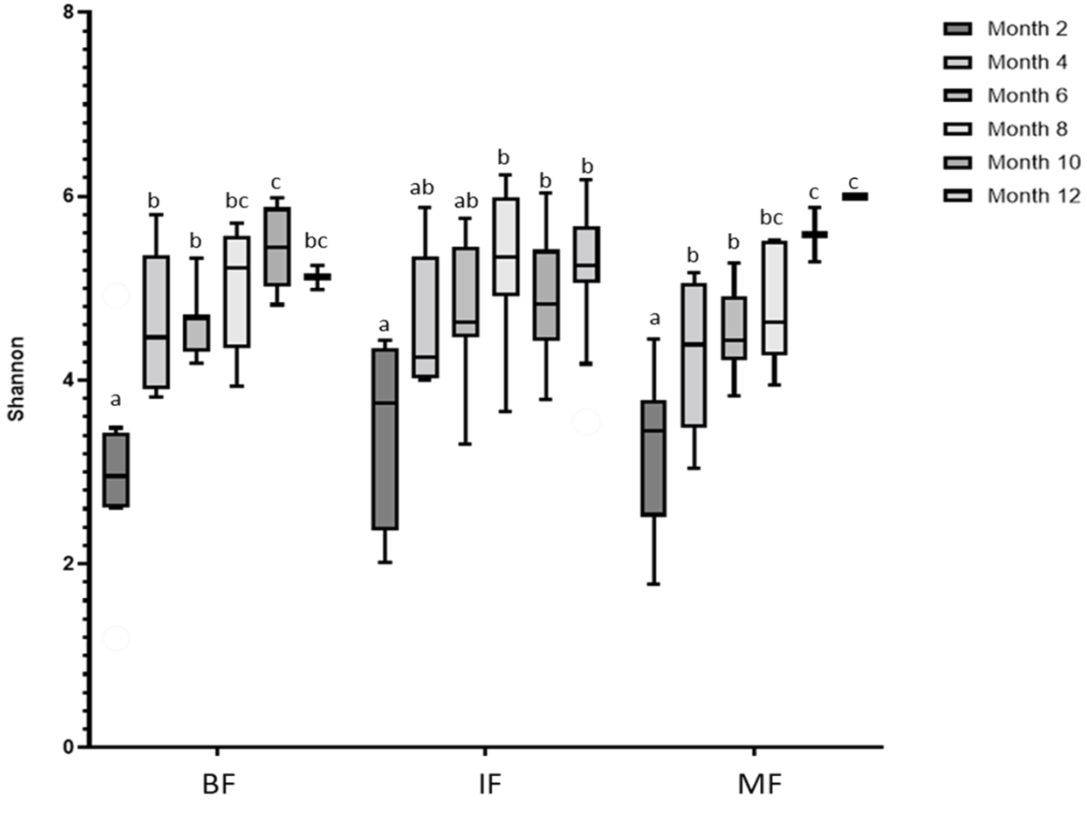


Figure 5. 1 Alpha diversity of infant gut microbiota during the first year of life. BF: infants with exclusively breastfeeding; MF: infants with mix feeding of breast milk and formula mil; IF: infants with exclusively feeding with formula milk.

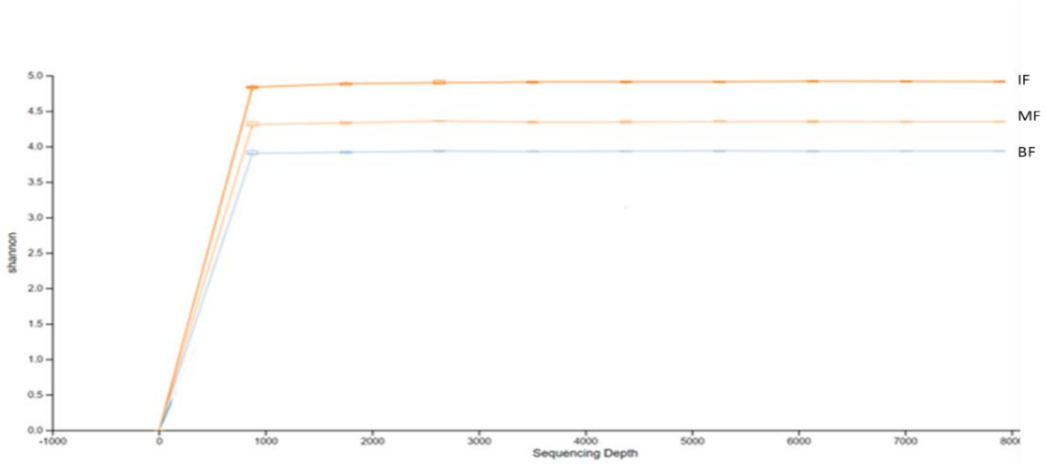
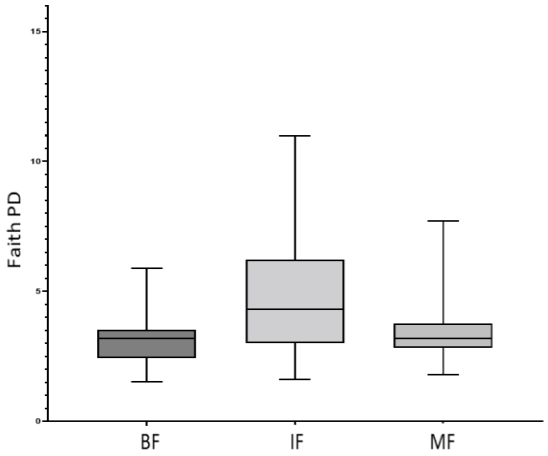


Figure 5 2 Alpha diversity of samples with different feeding modes
BF: infants with exclusively breastfeeding; MF: infants with mix feeding of breast milk and formula milk; IF: infants with exclusively feeding with formula milk.

(A)



(B)

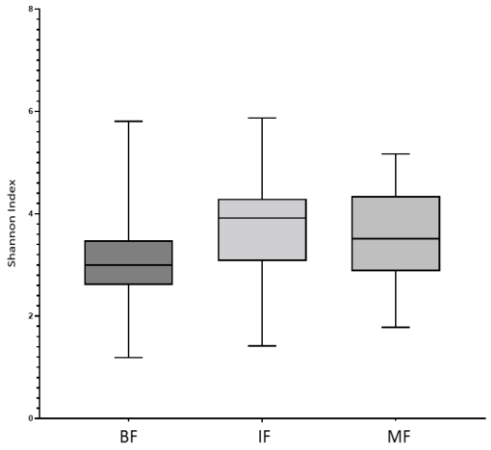


Figure 5.3 Alpha diversity of gut microbiota in infants of different feeding modes.
(A) Faith PD of fecal samples at month 2-4 of infant age. (B) Shannon index of fecal samples at month 2-4 of infant age. BF: infants with exclusively breastfeeding; MF: infants with mix feeding of breast milk and formula milk; IF: infants with exclusively feeding with formula milk.

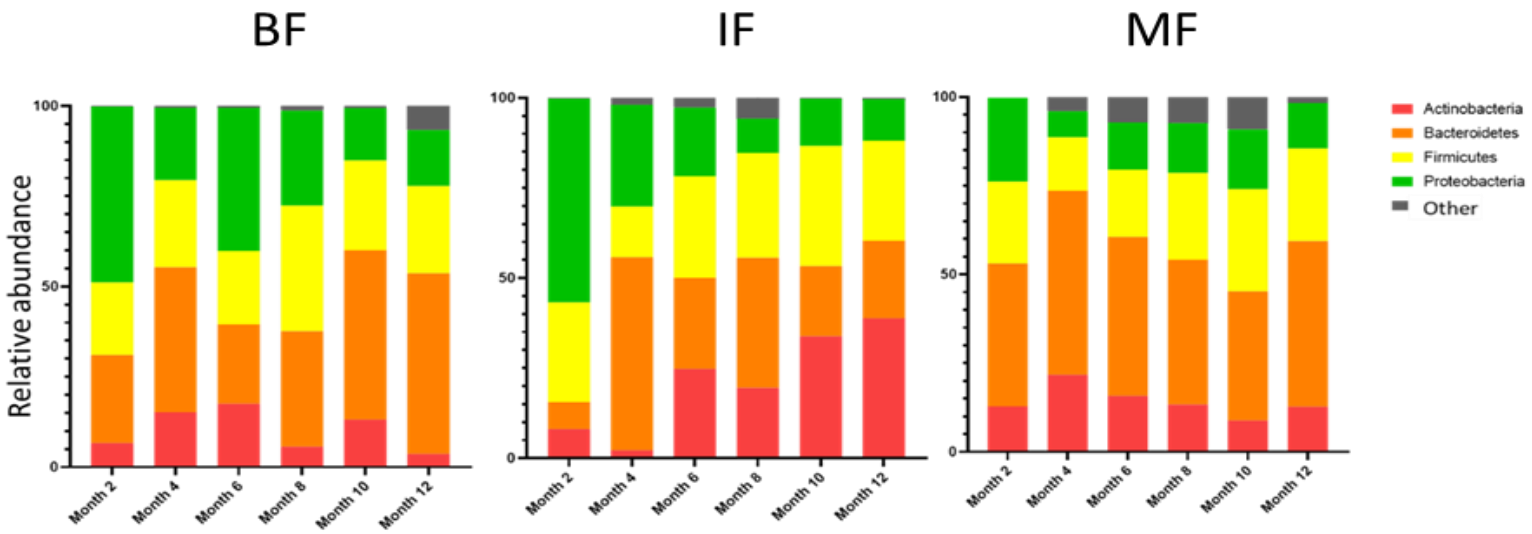


Figure 5.4 The gut microbiota at phylum level during the first year of life.
BF: infants with exclusively breastfeeding ; MF: infants with mix feeding of breast milk and formula milk; IF: infants with exclusively feeding with formula milk.

5.3.4 Effect of Feeding Mode on Infant Gut Microbiota at Phylum and Genus Level

The relative abundance of bacterial taxa at Phylum level of infant during the first 6 month of life was shown in Figure 5.5. The formula fed groups had lower abundance of Actinobacteria than the other two feeding modes at month 2 and month 4 which the infants were fed with breast milk and/or formular milk only. However, the observation disappeared from month 6 onward when solid food was commonly introduced. In addition, the phylum Bacteroidetes level was higher in IF group than BF group at month 4 ($p<0.05$) while a lower level of Proteobacteria in fecal samples of IF group than those breast-fed ($p<0.05$). Figure 5.6 shows the relative abundance of bacterial taxa at Genus level of infant during the first half year of life. BF group had a higher abundance of *Bifidobacterium* ($p<0.05$) at month 2 and month 4. A higher level of *Lactobacillus* was observed, despite statistical insignificance. However, this trend disappeared after month 6, implying the sources of these two beneficial bacteria being breast milk. The level of *Clostridium_sensu_stricto_1* was higher in the IF group than the other two feeding modes at month 6 ($p<0.05$).

5.3.5 Sickness and Infant Gut Microbiota

In addition to feeding modes, other factors may also influence the development of the infant gut microbiota. Fecal samples of 3 infants who were exclusively breastfed were analysed across the first year of life with the indication of solid food introduction and sicknesses such as cold, diarrhoea, fever, or viral infection. The results indicated that the *Bifidobacterium* level was comparatively low when the subjects got sick for example diarrhoea in month 8 of EPS007, Flu/fever in month 12 of EPS007 and month 8 of EPS0023 (Figure 5.7).

5.3.6 Correlation between Maternal Dietary Intake during Lactation and Infant Gut Microbiota at Month 2

In total, 26 pairs of mother-infants cohort were recruited. Two types of correlation test were used in the analysis: Pearson and Spearman correlation. Correlation study is to study the association between variables which are maternal nutrient intake and infant gut microbiota in our study. The result indicated that several nutrient intakes during lactation in mother correlated with the infant gut microbiota using Pearson correlation test (Figures 5.8-11A) and Spearman correlation test (Figures 5.8-11B), respectively. However, there are some differences between these two tests. Pearson correlation is normally for those data with a normal distribution and present linear relationship while Spearman correlation is for those samples which are non-normally distributed and can measure monotonic association. Pearson correlation is more sensitive to outliers than Spearman correlation (Schober et al., 2018). To test the if the data follow normal distribution, Shapiro-Wilk test were performed. It was found that the gut microbiota data were non normally distributed. Therefore, result of Spearman correlation's data was used to explain the result in this study.

Total fiber intake was positively correlated with the Family Tannerellaceae ($p<0.01$; Figure 5.8B), and Genera *Parabacteroides* ($p<0.01$; Figure 5.10B). Intake of total sugar, fructose, galactose and glucose ($p<0.05$; Figure 5.8B) were positively correlated with Family Enterobacteriaceae ($p<0.05$; Figure 5.8B). There was also a positive association between total sugar intake with Genera *Escherichia-shigella* ($p<0.05$; Figure 5.10B). The polyunsaturated fatty acid intake was found to have a positive association with Family Ruminococcaceae ($p<0.05$; Figure 5.9B). Intake of trans fat and some medium chain saturated fatty acid, including C10:0 and C12:0 was positively correlated with Genera *Escherichia-shigella* ($p<0.01$) while intake of polyunsaturated fatty acid

including C18:2, C18:4, C20:4 C20:5, C22:6, omega 6 and cholesterol was positively correlated with *Klebsiella* ($p<0.05$; Figure 5.11B). Some negative associations were observed between the monounsaturated fatty acid intake, polyunsaturated fatty acid and infant gut microbiota. Arachidonic acid (C20:4 omega 6) was negatively associated with Family Bifidobacteriaceae ($p<0.05$; Figure 5.9B) and Genera *Bifidobacterium* ($p<0.05$; Figure 5.11B). While maternal intake of C17:1, C18:3; Omega 6 and cholesterol were negatively correlated with Family Lactobacillaceae ($p<0.05$; Figure 5.9B) and Genera *Lactobacillus* ($p<0.05$; Figure 5.11B). Maternal intake of vitamin A, vitamin B1, B2 and folate during lactation were negatively correlated with infant gut microbiota such as *Streptococcus, Clostridium_sensu_stricto_1 and Veillonella* ($p<0.05$; Figure 5.12B). Infant gut *Enterococcus* were found to have negative correlation with maternal protein, fatty acids include C:10:0, C:12:0, C20:5 and C22:6, Vitamin B3, Calcium and Sodium intake during lactation ($p<0.05$; Figure 5.9-13B).

Regarding the food group intake during lactation, grain and vegetable intakes were negatively associated with *Lachnoclostridium* and *Veillonella* while dairy group intake was positively correlated with *Escherichia-shigella* ($p<0.05$; Figure 5.14B).

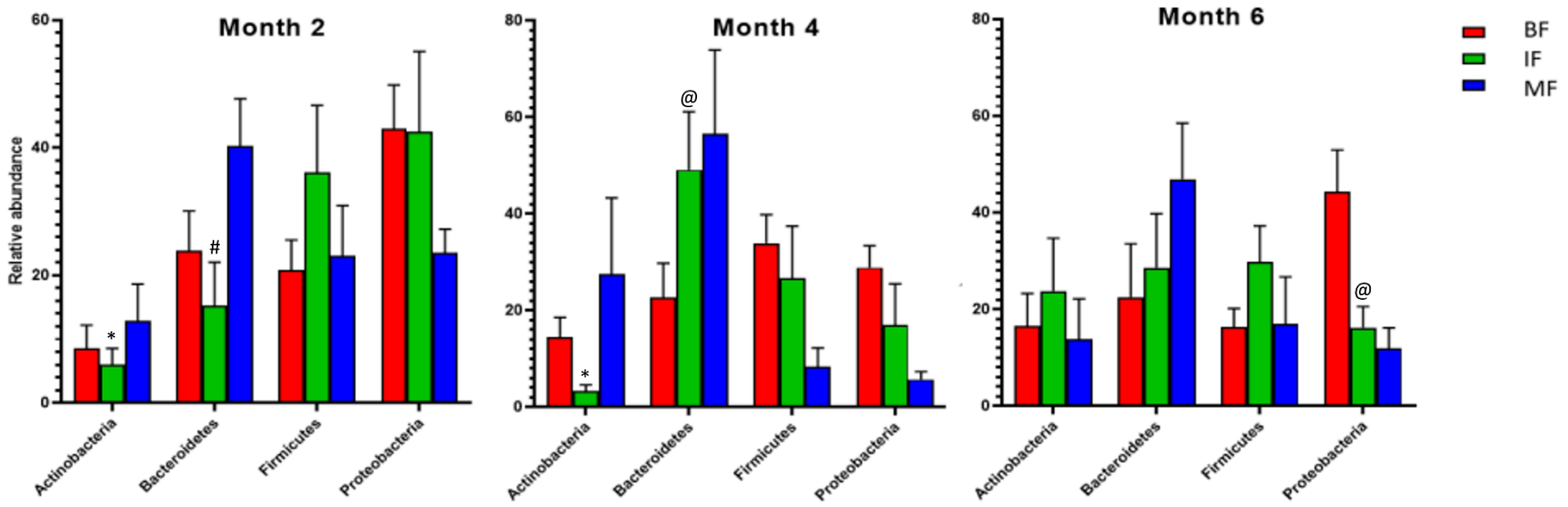


Figure 5.5 Relative abundance of bacterial taxa at phylum level of infant during the first 6 month of life.
BF: infants with exclusively breastfeeding; MF: infants with mix feeding of breast milk and formula milk; IF: infants with exclusively feeding with formula milk. * Significant difference vs BF group and MF group ($p<0.05$); # Significant difference vs MF grop($p<0.05$); @ Significant difference vs BF group ($p<0.05$).

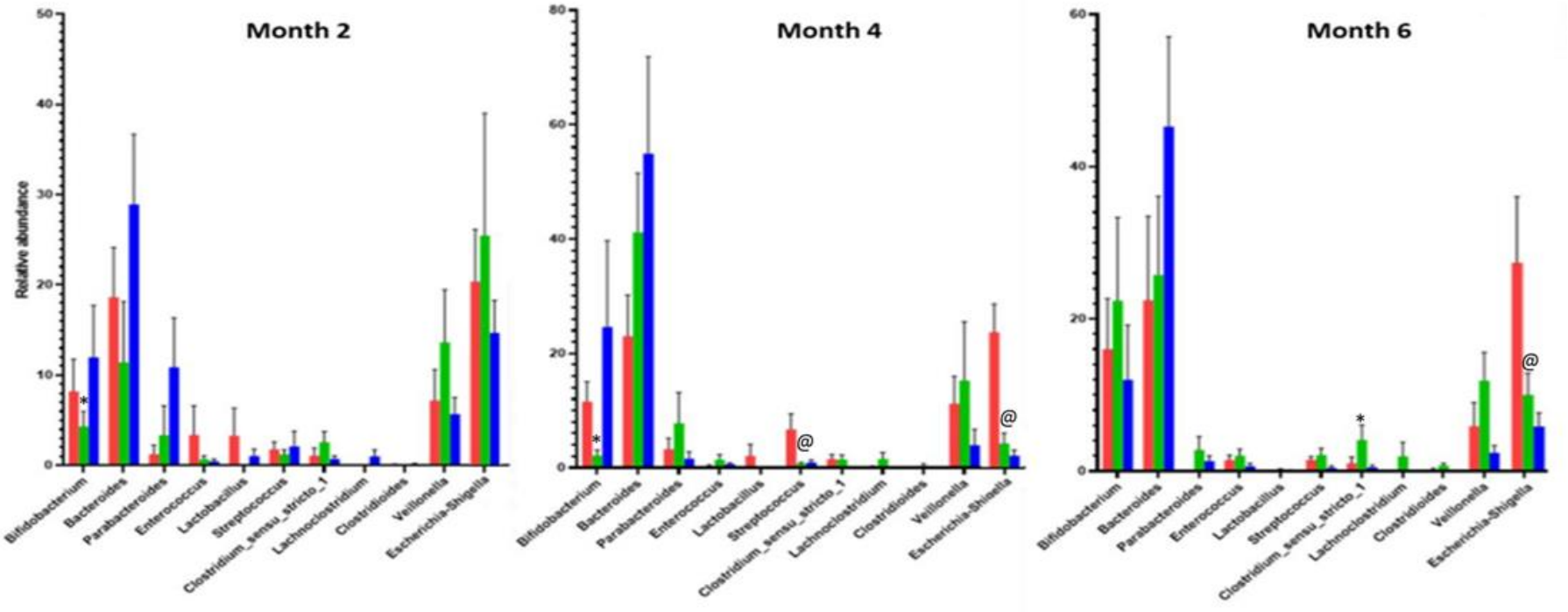


Figure 5.6 Relative abundance of bacterial taxa at genus level of infant during the first 6 month of life.
BF: infants with exclusively breastfeeding; MF: infants with mix feeding of breast milk and formula milk; IF: infants with exclusively feeding with formula milk. * Significant difference vs BF group and MF group ($p<0.05$); @ Significant difference vs BF group ($p<0.05$).

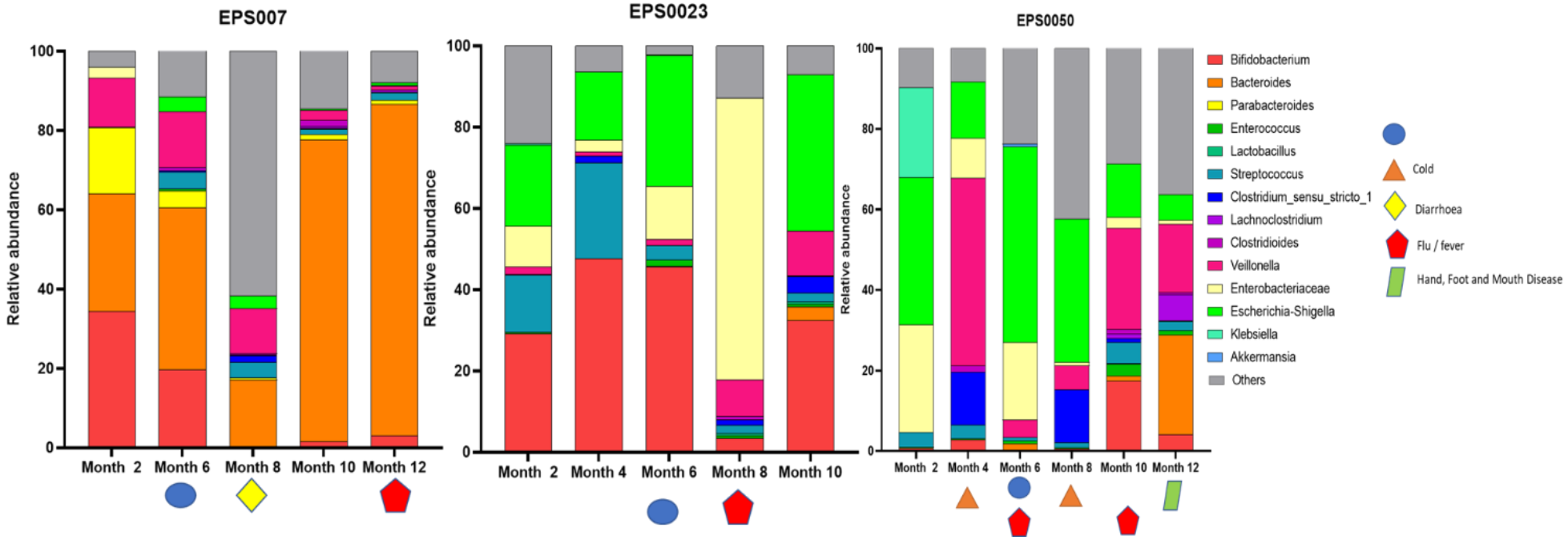
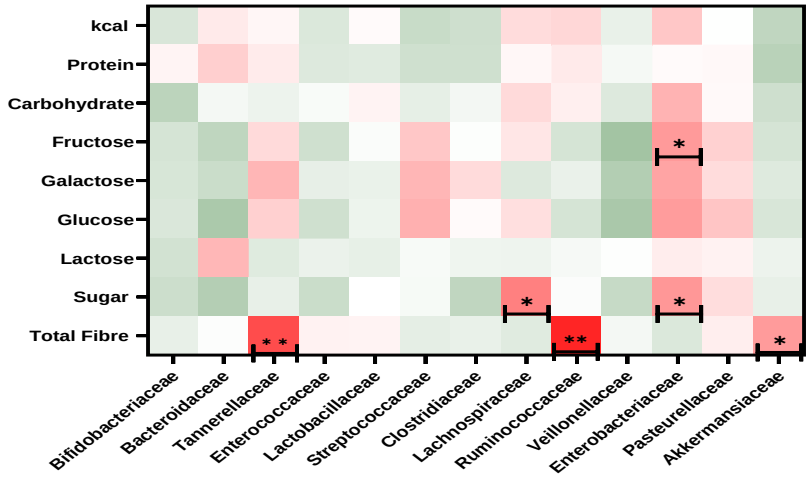


Figure 5.7 Relative abundance of bacterial taxa at genus level of infant during the first year of life of 3 individual infants who were exclusively breastfed.

(A) Pearson correlation



(B) Spearman correlation

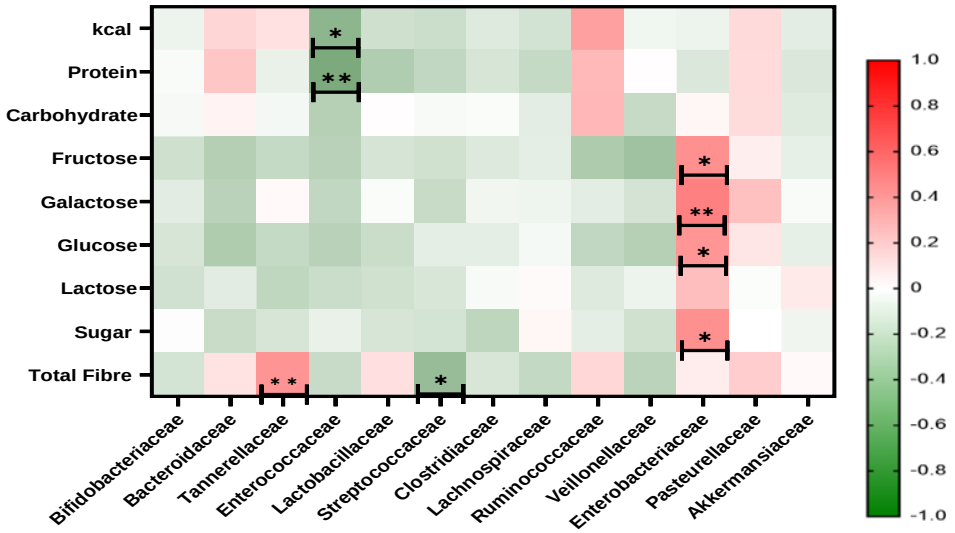
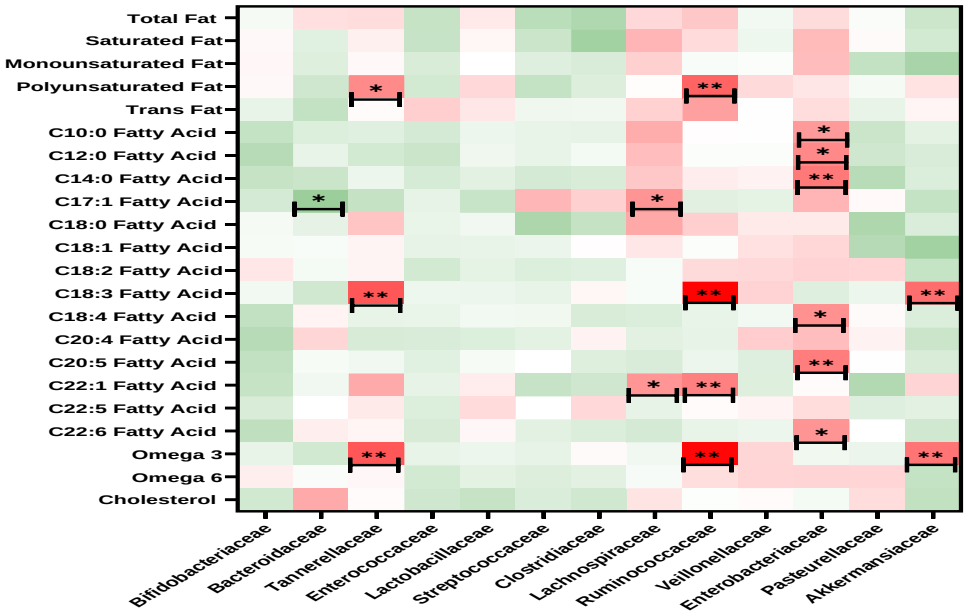


Figure 5.8 Correlation between maternal kilocalories, protein and carbohydrate intake during lactation with infant gut microbiota at family level.

(A) Pearson correlation (B) Spearman correlation. * and ** presents a significant level of < 0.05 and < 0.01 respectively. The red color indicates a positive correlation while green color indicates a negative correlation.

(A) Pearson ‘s correlation



(B) Spearman correlation

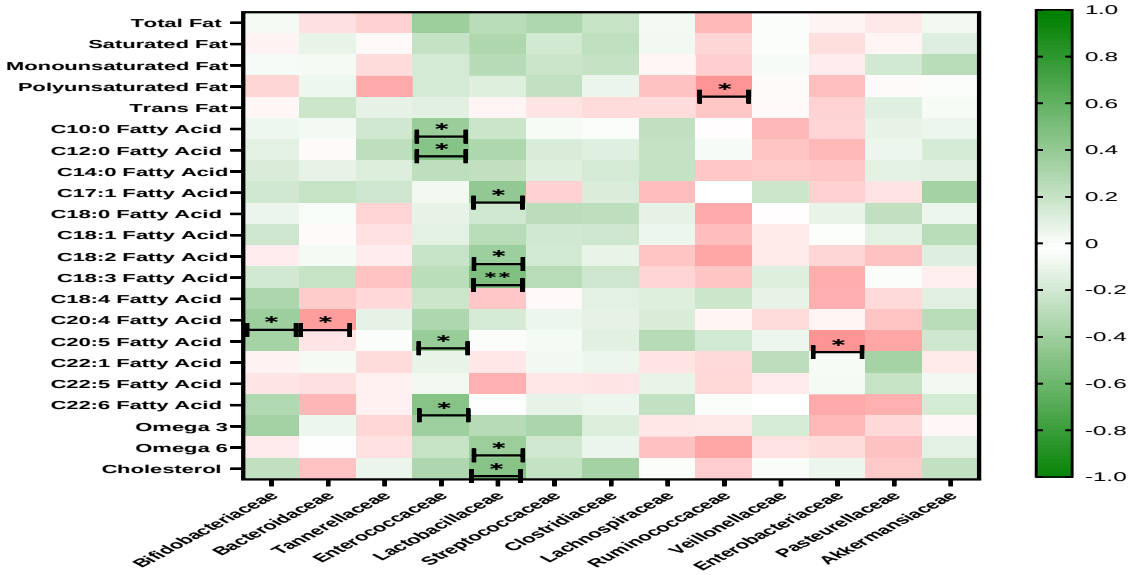
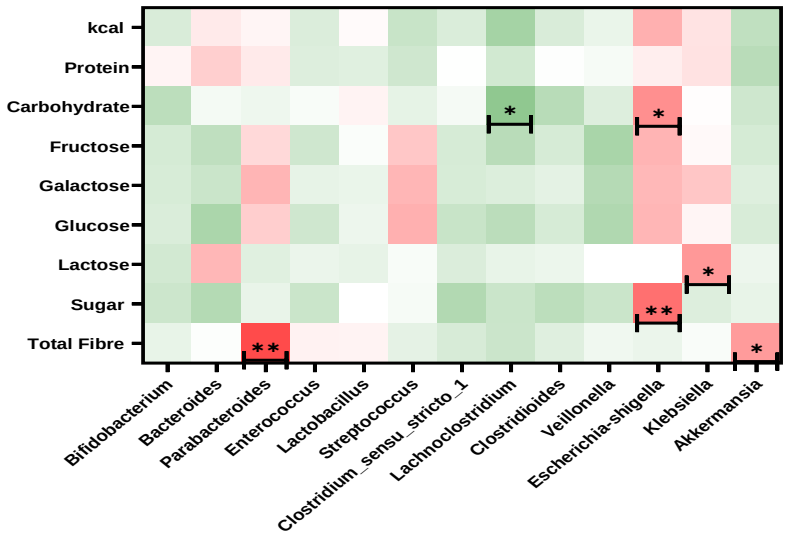


Figure 5.9 Correlation between maternal dietary fat diet during lactation with infant gut microbiota at family level.

(A) Pearson correlation (B) Spearman correlation. * and ** presents a significant level of < 0.05 and < 0.01 respectively. The red color indicates a positive correlation while green color indicates a negative correlation.

(A) Pearson correlation



(B) Spearman correlation

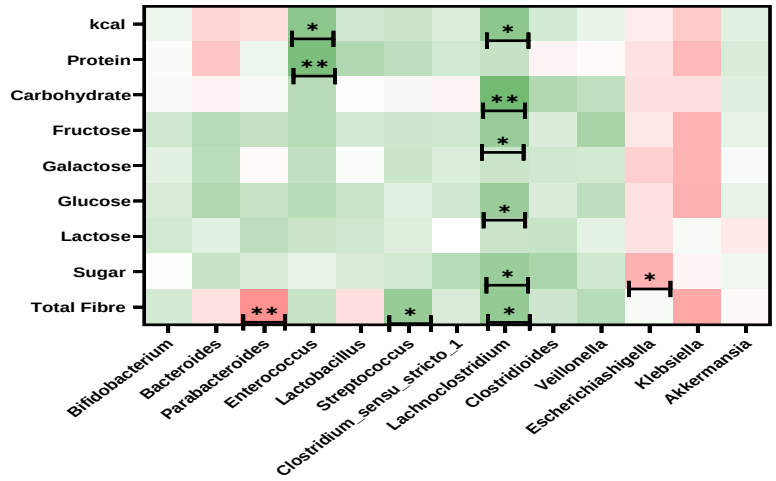
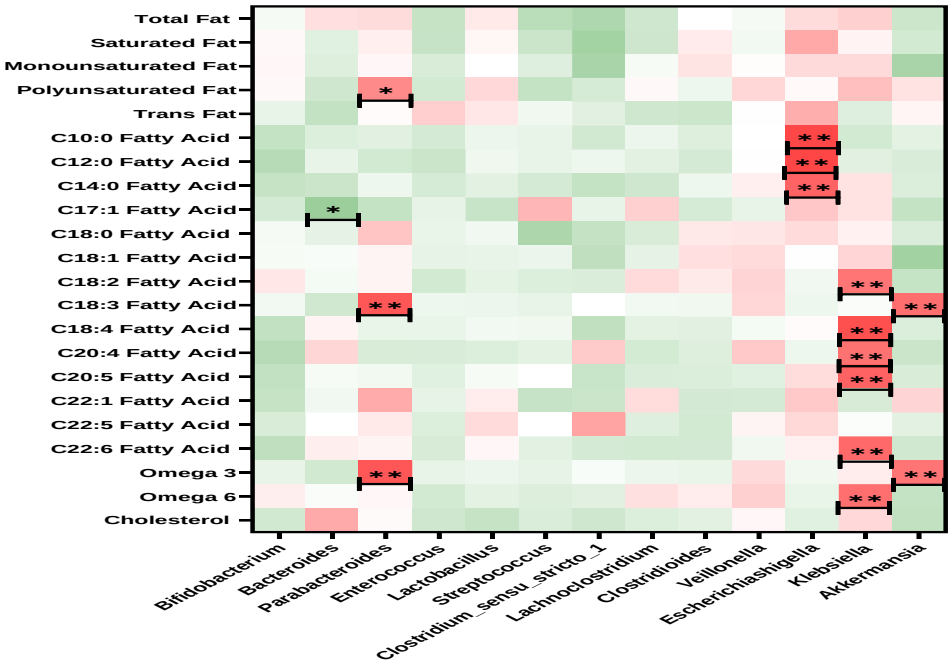


Figure 5.10 Correlation between maternal kilocalories, protein and carbohydrate intake during lactation with infant gut microbiota at genus level.

(A) Pearson correlation (B) Spearman correlation. * and ** presents a significant level of < 0.05 and < 0.01 respectively. The red color indicates a positive correlation while green color indicates a negative correlation.

(A)



(B)

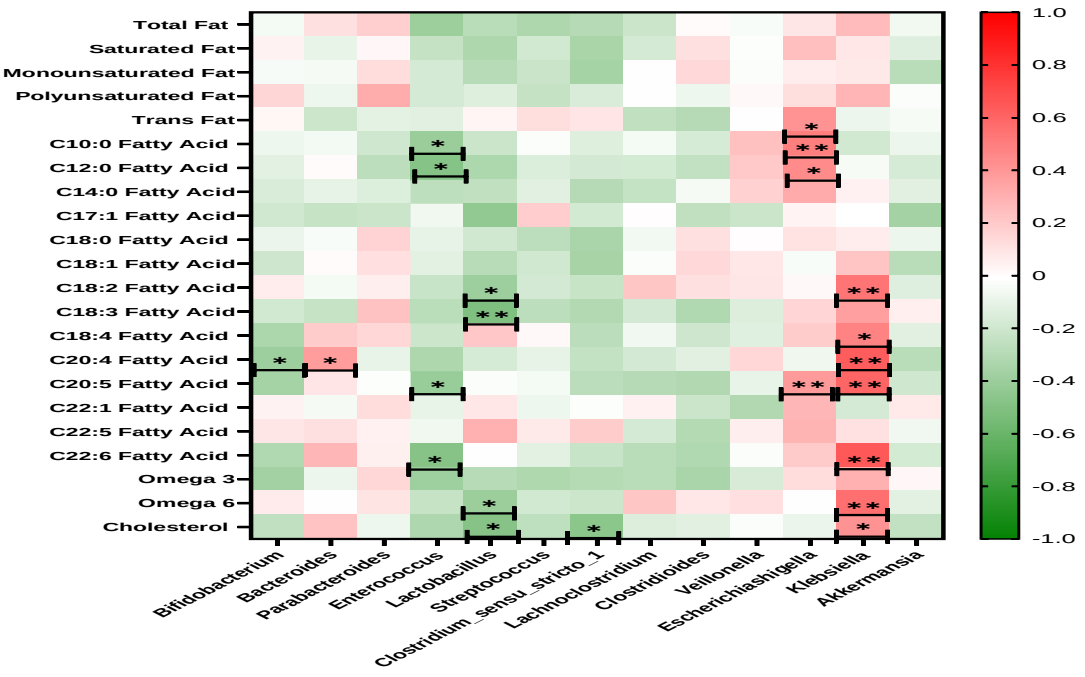
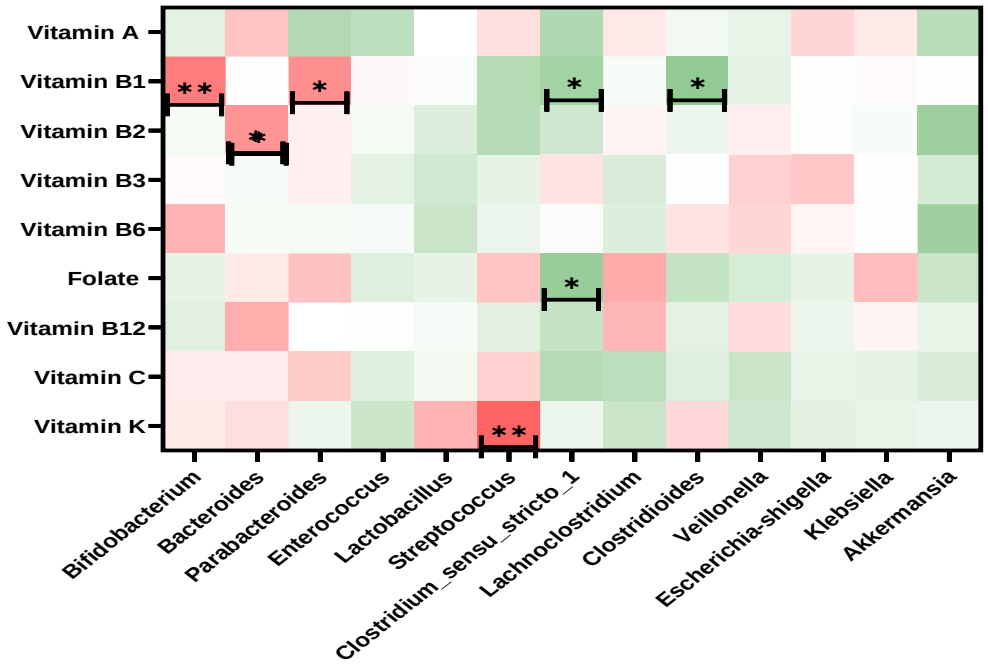


Figure 5.11 Correlation between maternal dietary fat diet during lactation with infant gut microbiota at genus level.

(A) Pearson correlation (B) Spearman correlation. * and ** presents a significant level of < 0.05 and < 0.01 respectively. The red color indicates a positive correlation while green color indicates a negative correlation.

(A) Pearson correlation



(B) Spearman correlation

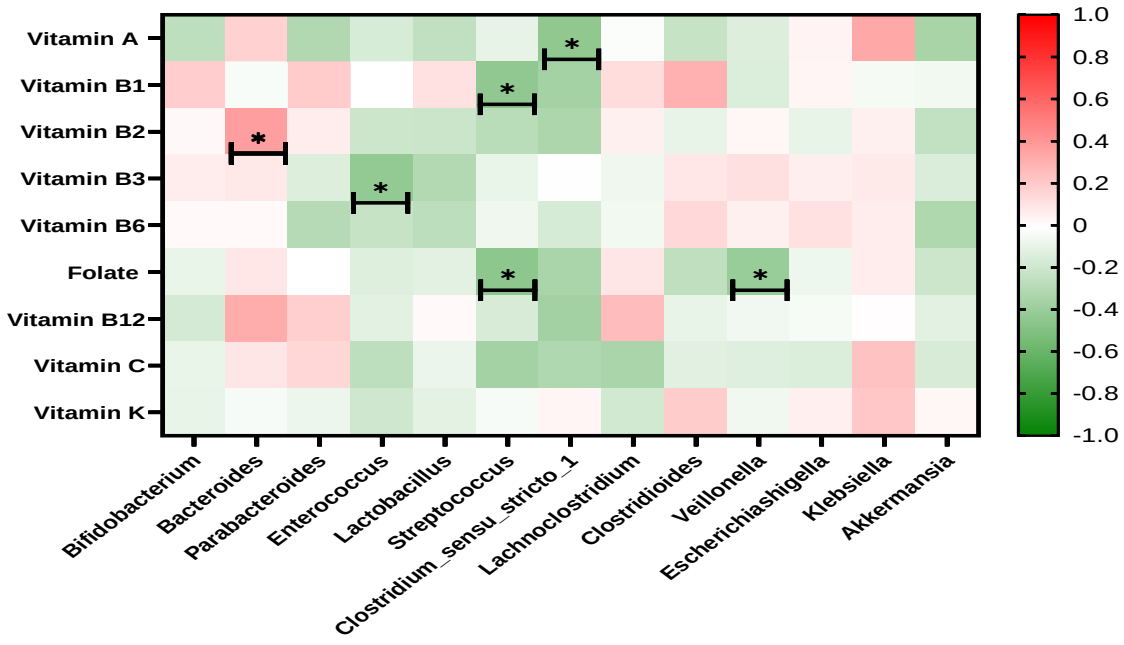
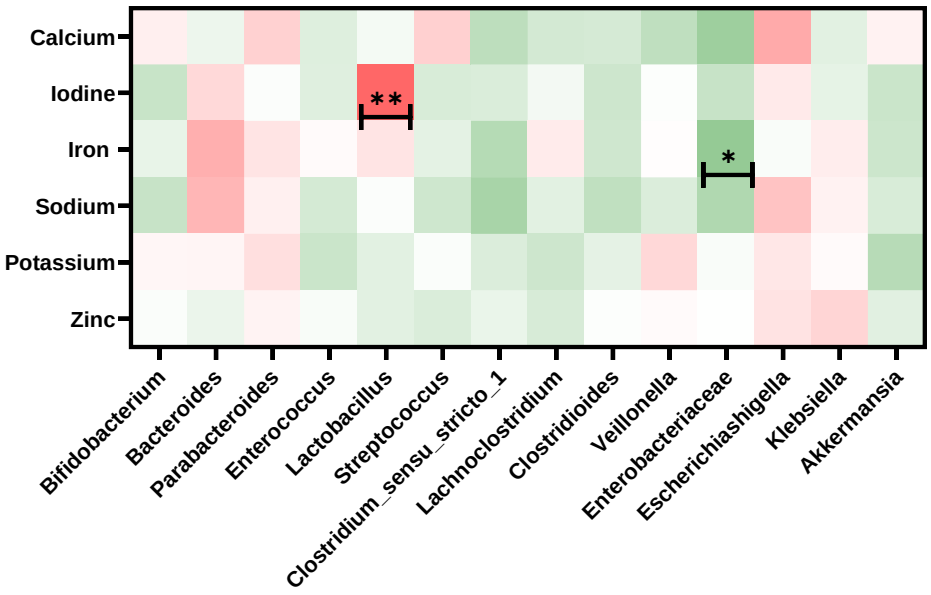


Figure 5.12 Correlation between maternal vitamin intake during lactation with infant gut microbiota at genus level.

(A) Pearson correlation (B) Spearman correlation. * and ** presents a significant level of < 0.05 and < 0.01 respectively. The red color indicates a positive correlation while green color indicates a negative correlation.

(A) Pearson correlation



(B) Spearman correlation

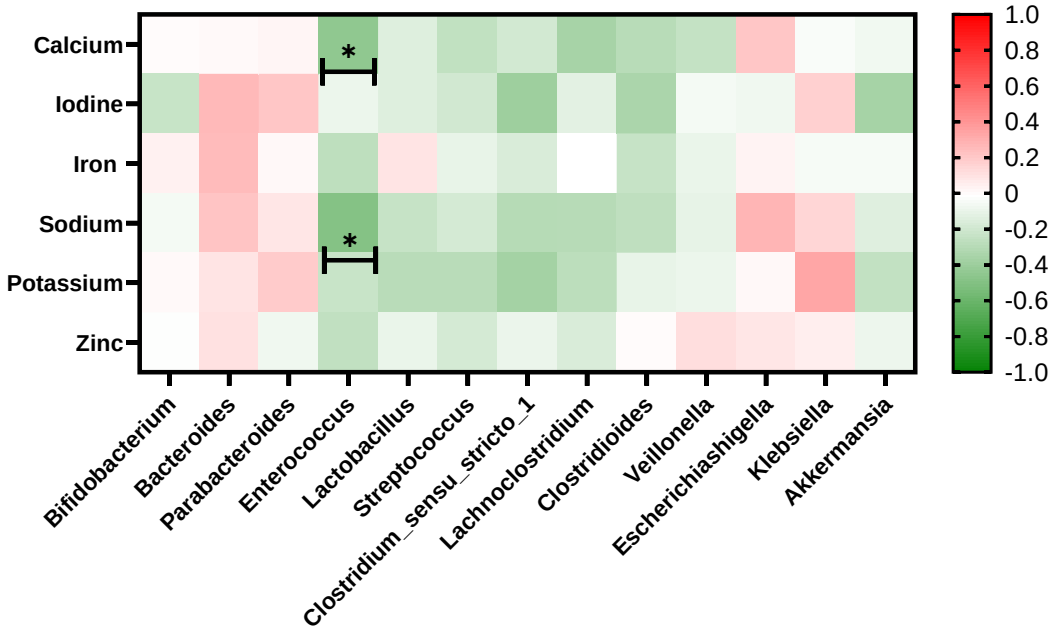
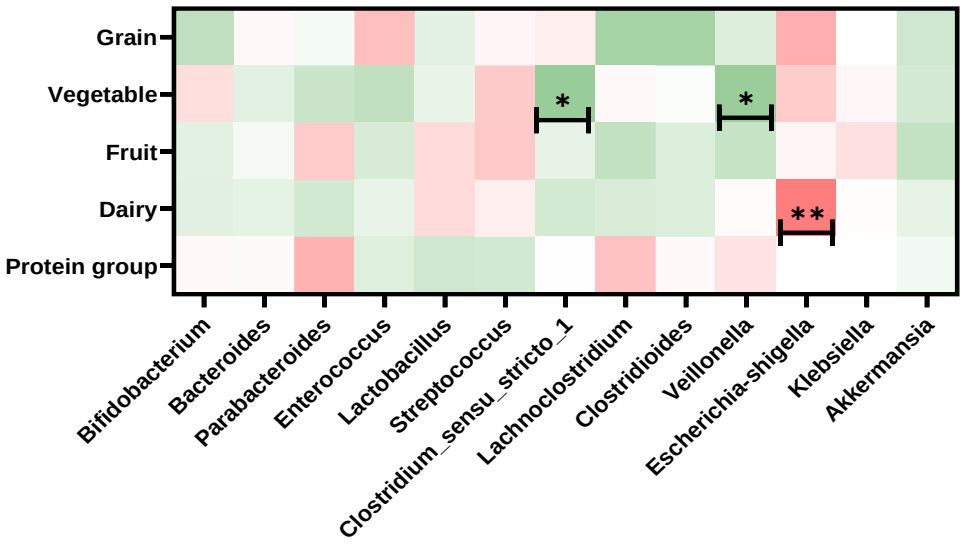


Figure 5.13 Correlation between maternal mineral intake during lactation with infant gut microbiota at genus level.

(A) Pearson correlation (B) Spearman correlation. * and ** presents a significant level of < 0.05 and < 0.01 respectively. The red color indicates a positive correlation while green color indicates a negative correlation.

(A) Pearson correlation



(B) Spearman correlation

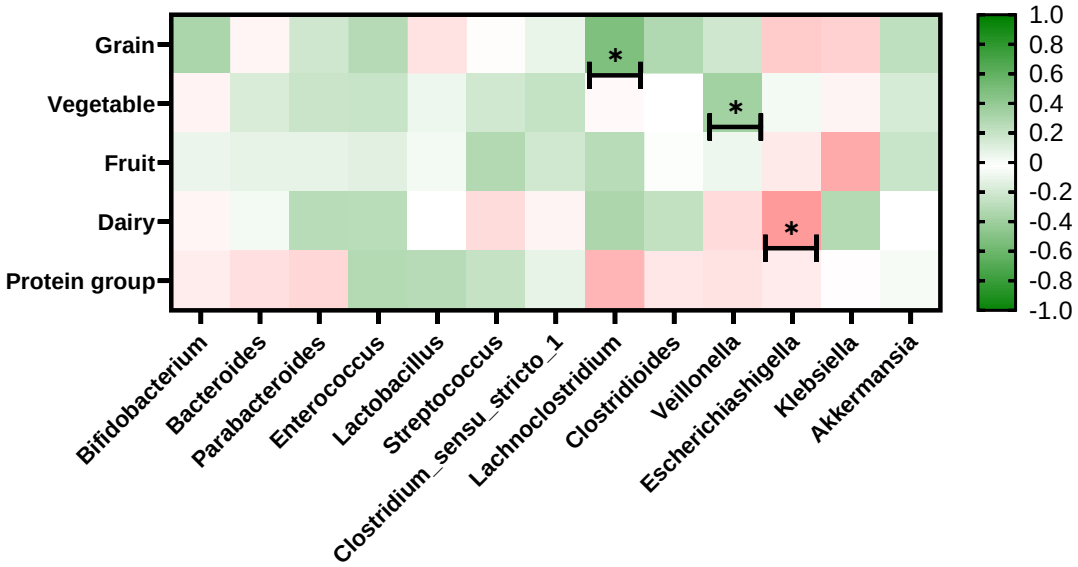


Figure 5.14 Correlation between maternal food group intake during lactation with infant gut microbiota at genus level.

(A) Pearson correlation (B) Spearman correlation. * and ** presents a significant level of < 0.05 and < 0.01 respectively. The red color indicates a positive correlation while green color indicates a negative correlation.

5.4 Discussion

A handful of studies have shown that gut microbiota is associated with health and various disease status. Understanding the development of gut microbiota in Hong Kong infants and the factors affecting the development may help to improve the health of the next generation. Since there is not much data in Hong Kong, the aim of this study is to determine the gut microbiota development of Hong Kong infants during the first year of life and examine if infant gut microbiota is shaped by maternal diet and early feeding practices.

Our study showed that Proteobacteria, Firmicutes and Bacteroidetes were the three predominant Phyla within the fecal samples across the first year of life. This observation was in line with the study of Chinese infants recruited in Shanghai (Niu et al., 2020). Proteobacteria level was the highest in month 2 while Bacteroidetes and Firmicutes became more dominant after month 4. This result was consistent with the finding reported by Gilley et al. (2022). In general, gut microbiota in newborn is dominated by Enterobacteriaceae (Proteobacteria phylum) due to the predominantly aerobic environment at birth, then the gut becomes anaerobic gradually and strict anaerobes can start colonization (Niu et al., 2020). After the introduction of solid food, more adult-type bacteria mainly the genera *Bacteroides*, *Ruminococcus*, and *Clostridium* etc., colonize in the infant gut increasingly and gradually become predominant (Tanaka & Nakayama, 2017). Our study showed that the alpha diversity was lower in month 2 when compared to the other months($p<0.05$), suggesting a less complex microbial community in the early stage of infant gut microbiota. These observations were consistent with other studies showing that the alpha diversity of the infant gut microbiota increased with age (Gilley et al., 2022; Hill et al., 2017; Niu et al., 2020).

Feeding mode is another critical factor affecting the infant gut microbiota after birth. A lower level of alpha diversity in BF group was observed than IF group at month 2 however it was not statistically significant, and the values were similar at month 4 and onward, indicating the influence of feeding mode on the gut microbial complexity is most impactful at early stage of gut microbiota maturation. Higher Genera *Bifidobacterium* were found in the fecal samples of BF group at month 2 and month 4 however this observation disappeared from month 6 when weaning started. A study conducted on infants aged 4-5 months in USA shared similar observation (Odiase, 2022). One of the reasons attributed to this observation may be due to the presence of HMO which is reported to harbor bifidogenic effect and favors the growth of *Bifidobacterium* spp. (Tanaka & Nakayama, 2017) and some live bacteria in human milk, which can pass to infant gut through breast milk (Solis et al., 2010).

In our study, even though some infants in IF groups were fed with formula supplemented with HMO, we observed a high level of *Clostridium_sensu_stricto_1* in IF group at month 6 besides the difference in the relative abundance of *Bifidobacterium* in early stage of breastfeeding. Similar finding was reported by Ruiz-Ojeda et al., who reported that babies fed with standard infant formula had higher *Clostridium* than the group with exclusively breastfed (Ruiz-Ojeda et al., 2023). The effect of feeding mode became less variable when age increased as compared to month 2 and month 4, the earlier life stages of the infants. This result could be due to introduction of solid foods which initiated the changes in the gut microbiota and gradually reduce the profound effect of breastfeeding (Moore & Townsend, 2019).

There is not much study on the development of gut microbiota of infants in Hong Kong particularly with multiple time points throughout the first year of life. Despite the sample size was relatively small, the result could give an overview of the development of Hong Kong population. Moreover, this study confirmed that most of the observation were similar to the study conducted in China as well as other regions.

Another objective of this study is to determine if maternal diet during lactation affects the gut microbiota developments in Hong Kong infants. We identified correlations between different nutrient intake and infant gut microbiota. Total fiber intake of maternal diet was positively correlated with the Family Tannerellaceae, which could utilize indigestible polysaccharides to produce short chain fatty acid propionate (Polansky et al., 2015) and butyrate (Poeker et al., 2018). Even though the maternal dietary fiber intake will not affect the dietary fiber content in milk much, Selma-Royo et al. (2022) revealed that insoluble fiber consumption increased fucosyllacto-N-hexaose (FLNH) level in the breast milk of secretors. They concluded that fiber, namely indigestible polysaccharides, was a factor affecting the HMO concentrations of breast milk from secretors.

Some medium chain saturated fatty acid intake and trans-fat intake was positively correlated with genus *Escherichia shigella* while polyunsaturated fatty acid intake was positively correlated with *Klebsiella*. To our best knowledge, there is no previous study to investigate the impact of maternal diet during lactation on infant gut microbiota with a comprehensive analysis of maternal diet. Only few studies about the impact of maternal dietary fat on the infant gut microbiota were done by different groups. It was reported that fatty acid profile in human milk is affected by the maternal fat intake

during lactation, which the fat in milk will affect the microbial composition of infant gut microbiota (Aumeistere et al., 2019).

Association between sn-2 fatty acid in milk and infant gut microbiota was found, for example, some saturated fatty acid such as myristic acid (C14:0), stearic acid (C18:0), and DHA showed correlation with Enterobacteriaceae (Jiang et al., 2018). In the case of adult cohort, clinical study showed that dietary fat intake could modulate the bacterial composition in gut and their metabolites. Saturated fatty acid and omega 6 fatty acid induced a high level of Enterobacteriaceae, an important and large family of Gram-negative bacteria (Alcock & Lin, 2015). Some animal studies also showed that high fat diet increased *Escherichia* and *Klebsiella* level in gut (Beam et al., 2021). In our study, fatty acids of maternal diet during lactation including some medium chain fatty acids C10:0, C12:0, and PUFA such as C18:2, C18:4, C20:4, C20:5, C22:6, and omega 6, showed positive correlation with Genera *Escherichia-shigella* and *Klebsiella* respectively, both of which are commonly considered harmful.

Omega 3 fatty acid intake was reported to prevent the dysbiosis induced by omega 6 fatty acid intake (Ghosh et al., 2013). Our study also indicated that the maternal total omega 3 intake did not have positive correlation with genus *Escherichia and Klebsiella* as other fatty acid or total omega 6 fatty acid intakes.

Lipopolysaccharide (LPS) is a component of the outer membrane of Gram-negative bacteria (Minihane et al., 2015) including family Enterobacteriaceae and genus *Escherichia and Klebsiella*. High-fat diet increased the relative abundance of LPS-containing microbiota in the gut (Cani et al., 2007) which is link with chronic low-grade

inflammation because LPS activates Toll-like receptor 4 (TLR4) and its binding of with TLR4 induces inflammatory response. Chronic low-grade inflammation has also been linked to higher risk of insulin resistance and Type II diabetes (Beam et al., 2021).

In our study, we also found out that some maternal polyunsaturated fatty acid intake during lactation such as arachidonic acid (a type of omega 6 fatty acid which could be obtained from food such as meat and poultry),α-linolenic acid and total omega 6 were negatively associated with probiotic species in infant gut such as *Bifidobacterium and Lactobacillus*. A study conducted by Kankaanpaa et al. (2001) indicated that polyunsaturated fatty acid intake may affect the adhesion and growth of probiotic species. Since the fatty acid intake in mothers will influence the fatty acid composition in breastmilk so it may also affect the infant gut microbiota development.

Maternal intake of vitamin B1, B2 and folate during lactation were correlated with infant gut microbiota such as *Streptococcus*, *Clostridium_sensu_stricto_1* and *Veillonella*. It is known that some gut microbes could produce vitamin Bs while some may compete for vitamin B with the host. The supplementation of Vitamin B or its deficiency could affect the growth of specific bacteria (Wan et al., 2022).

To sum up, this is one of the very first study to illustrate the relationship between the maternal diet during lactation and gut microbiota in Hong Kong infants. The result of this study indicated that maternal dietary intake especially dietary fiber, fat and vitamin Bs intakes were associated with the infant gut microbiota. Moreover, the effects of maternal diet during lactation on infant gut microbiota may either through changing the HMO or the human milk microbiota content in breast milk. It is worth examining the

breast milk content to understand the mechanisms behind. However, there were some limitations in this study include low sample size and no multiple testing correction conducted to adjust the result and avoid the false positives. Although there were limitations, the result provides implication that the development of infant gut microbiota could be shaped by adjusting the diet of lactating mother during breastfeeding. In short, the result of study is meaningful and sheds light for more large-scale study in the future.

Chapter 6 Association Between Maternal Diet, Human Milk Oligosaccharides, and Infant Gut Microbiota

6.1 Introduction

Exclusively breastfeeding for the first six months of life is recommended by World Health Organization (WHO, 2017). Breast milk is a natural ideal food source for infants because it contains a balance of nutrients and bioactive ingredients, for example, immunoglobulins, hormones, and oligosaccharides (Wicinski et al., 2020).

Human milk contains human milk oligosaccharides (HMO). HMO are important bioactive components and this kind of carbohydrates are the fourth most abundant compound group in human milk after water, lipids and lactose (Walsh et al., 2020). The concentration of HMOs in mature milk ranges between 5-15 g/L (Bode, 2012). Even though HMOs cannot directly provide nutrition to the infant, they will arrive the large intestine and readily be used by the gut microbiota (Jost et al., 2015). Therefore, it has been widely proposed that breast milk will bring health benefits to infants through altering the development of the infant gut microbiota.

Up to date, there are more than 200 different types of HMO being identified (Ballard & Morrow, 2013; Wu et al., 2017) which the concentration seems to be dynamic throughout lactation period (Soyyilmaz et al., 2021). There are three major types of HMOs, namely which ‘Acidic sialylated HMO’ for example 2 '-sialyllactose (2 'SL), ‘Neutral Fucosylated HMO’ such as 2 '-Fucosyllactose (2′FL) and ‘Neutral N-containing HMO’ including lacto-N-tetraose (LNT) and lacto-N-neotetraose (LNnT) (Wicinski et al., 2020). Among these groups, neutral HMOs account for more than 75% of the total HMOs in human milk (Totten et al., 2012; Vandenplas et al., 2018) 2 'FL

and LNnT are two important neutral HMOs because 2 'FL is by far the most abundant HMOs in 'secretor' mothers and LNnT is considered to co-regulate the synthesis of 2 'FL (Sprenger et al., 2017; Vandenplas et al., 2018). Sialylated HMOs which compose 12-14% of the total HMOs and 3'-sialyllactose (3'SL) and 6'-sialyllactose (6'SL) are two predominant forms of sialylated HMOs. Both of them are reported to have anti-inflammatory and bifidogenic effects (Simon et al., 1997; Yu et al., 2013). In this study, we analyzed the HMO content in the breast milk throughout the 1st year of lactation.

Although HMOs cannot directly provide nutrition to the infant, they are found to assist in shaping the infant gut microbiota, early-life immune development and preventing against infectious diseases (Doherty et al., 2018; Walsh et al., 2020). Different factors may affect the composition of HMO in human milk. Lactating mother with genes encoding for galactoside 2-α-L-fucosyltransferase 2 *(FUT2)* and galactoside 3-α-L-fucosyltransferase 3 *(FUT3)* (secretor genes) produces more diverse HMO when compared with those of non-secretors and the total concentration of HMO has been shown to be significantly higher in "secretor" human milk when compared with "non-secretor" human milk (Kunz et al., 2017). Some research studies suggested that maternal diet also affects HMO composition (Ferreira et al., 2020; Seferovic et al., 2020). However, the relationship between maternal diet and HMO composition still needs additional research studies to confirm the linkage. In this study, correlation between maternal diet with HMOs content in breast milk was examined.

6.2 Methodology

Mothers were recruited using an opportunity sampling method and screened by our research staff to check the eligibility. A 3-day diet record was used to assess the diet

habit of mothers during lactation. Each mother was asked to provide mature milk samples (30-40 ml) in the same week when diet record and infant fecal samples were provided. HMOs were extracted from the samples and undergone Liquid chromatography-mass spectrometry (LC-MS) analysis using HMO standard. Data analysis was performed in the software Statistical Package for Social Sciences (SPSS), version 29.0 (Chicago, USA). Descriptive statistics were generated for characteristics of maternal diet during lactation and HMO concentration. ANOVA analysis with post hoc Ducan test was used to determine the difference between different types of HMO in the same month and between same HMO across the lactation period (p<0.05). Pearson correlation tests to check for association between the nutrient intake during lactation and HMO in human milk. While both Pearson correlation and Spearman correlation were used to test the association between HMO in breast milk and infant gut microbiota. p<0.05 was reported as trends.

6.3 Results

6.3.1 HMO Concentration Throughout the Lactation Period

In total, 59 milk samples were analyzed from 2, 4, 6, 8, 10, and 12 months after birth from 28 subjects. The top 5 most abundant HMOs were identified in the breast milk samples, which were 2′FL, LNnT, 6′SL, 3′SL, and 3-fucosyllactose (3FL). The most abundant HMO throughout the whole lactation period in mature milk was 2′FL which was in average, 1048.0 ± 141.6 ppm in 2-month breast milk samples (Table 6.1). Overall, the HMO concentration was dynamic throughout the lactation period, and the concentration of 2′FL decreased from 2 months (1048.0 ± 141.6 ppm) to 12 months (475.2 ± 345.5) of lactation period, although it is not statistically significant. LNnT concentration was the highest in the milk of 2 month and its concentration reduced after

that ($p<0.05$). Total HMO concentration reduced throughout the lactation period ($p<0.05$) (Figure 6.1).

6.3.2 Correlation between Maternal Diet during Lactation and HMO Concentration in Breast Milk

Correlation analysis between the maternal diet during lactation at month 2 and HMO concentration in milk from 19 samples was conducted. The intake of macronutrients, including carbohydrates, protein, and fat, supply energy for mothers to produce breast milk. The monosaccharides such as glucose and galactose are the building block of HMO, while almost all HMOs are with lactose at the reducing end. Therefore, the correlation between glucose, galactose, lactose, and HMO presence in breast milk is worth investigating (Figure 6.2). In addition, dietary fiber includes insoluble and soluble dietary fiber, which are indigestible by human digestive enzymes but can be utilized by beneficial bacteria in the gut. The total dietary fiber intake was found to be inadequate during lactation in our study, so it is also meaningful to examine if the fiber intake in mothers is associated with the HMO content in breast milk (Figure 6.2).

Fat in our diet includes unsaturated and saturated one. Saturated fat intake is associated with a higher risk of cardiovascular diseases when compared with unsaturated fat. However, intake of trans fat and omega 6 fatty acids, type of unsaturated fatty acid, is associated with a higher risk of inflammation in the body when compared to other unsaturated fatty acids such as omega 3. The result of the association between different types of fat intake and HMO content in breast milk is shown in Figure 6.3.

To support babies' growth and mothers' health, adequate intake of different

micronutrients is essential. Micronutrients include vitamins and minerals. Correlation analysis was carried out between Vitamin A, B complex, C, K, calcium, iron, sodium, potassium, zinc, and iodine intake with HMO concentration. Figures 6.4A and 6.4B show the vitamin and mineral results, respectively.

A healthy balanced diet during lactation should include a suitable number of servings of grain, vegetables, fruit, protein group, and dairy products because each food group supplies different nutrients to mothers. The inadequacy may affect the biosynthesis of breast milk content, including HMO. Figure 6.5 illustrates the association between HMO content and maternal intake during each food group lactation.

The result showed that LNnT was positively correlated with maternal dietary intake of carbohydrate, lactose and dietary fiber, including both soluble dietary fiber and insoluble dietary fiber. Positive association was also found between the total dietary fiber and insoluble dietary fiber intake in mothers during lactation and the total HMO presence in milk (Figure 6.2). 2'FL was negatively correlated with energy intake, while no correlation could be identified between the intake of macronutrient, dietary fiber, and 6′SL, 3′SL, and 3FL (Figure 6.2). Figure 6.3 shows that total fat and saturated fat intake, including C14:0 and C18:0, were positively correlated with 3′SL. A higher intake of C18:3 and total omega-3 fatty acids was associated with more LNnT in human milk. In contrast, two fatty acids C17:1 and C18:0 intake were negatively associated with 2′FL. Positive correlations were observed between LNnT concentration and micronutrients, including vitamin B1, B2, B6 and folate. No other significant correlation could be detected between fat intake and 6′SL, 3FL, and total HMO in breast milk (Figure 6.4A). Iron and Zinc intake were negatively correlated with 2′FL, while

iron and iodine were positively associated with LNnT (Figure 6.4B).

Potential associations between maternal food group intake and HMO concentration were explored. Grain intake and fruit intake were positively correlated with LNnT and 2′FL, respectively, while intake of the protein group was found to have a significant positive correlation with 6'SL and 3′SL in human milk (Figure 6.5).

6.3.3 Correlation between HMOs and Infant Gut Microbiota at Genus Level

There are both beneficial bacterial and harmful microbes in the gut. At the genus level, well-known beneficial bacteria include *Bifidobacterium, Lactobacillus, Streptococcus, and Akkermansia,* and opportunistic pathogens include *Escherichia-Shigella* and *Klebsiella* which are gram-negative bacteria. HMO in breast milk can be utilized by beneficial bacteria in the infant's gut. The growth of beneficial bacteria may outcompete and inhibit the growth of pathogenic bacteria.

Total 18 pairs of breast milk and infant gut fecal samples were analyzed. Both Pearson and Spearman correlation were used to analyze the correlation. Not much correlation with statistical significance between the two was observed. Since the infant microbiota data were not normally distributed, the result of Spearman correlation is described and discussed. There was positive correlation between HMO in breast milk and *Veillonella*, *Parabacteroide*, and *Akkermansia* in fecal samples of infants. *Veillonella* was positively correlated with the 2′FL while *Parabacteroides* and *Akkermansia* were correlated with 3FL in the breast milk. *Akkermansia* was also positively associated with total HMO in the breast milk. (Figure 6.6). Two negative association were observed which was *Bacteroides* and *Enterococcus* with 3'SL and 2'FL, respectively.

Table 6. 1 Concentration of HMO throughout lactation period

Month	2-month (ppm) n = 20	4-month (ppm) n = 9	6-month (ppm) n = 11	8-month (ppm) n = 8	10-month (ppm) n = 7	12-month (ppm) n = 4
2'FL	1048.0 ± 141.6	949.5 ± 242.3	792.0 ± 162.1	637.7 ± 167.8	630.8 ± 173.2	475.2 ± 345.5
LNnT	813.4 ± 159.8	407.5 ± 87.7	261.9 ± 40.6	284.3 ± 63.6	329.9 ± 46.5	132.6 ± 88.4
6'SL	2.1 ± 0.5	1.4 ± 0.4	0.3 ± 0.2	0.0 ± 0.0	0.5 ± 0.2	0.4 ± 0.4
3'SL	4.9 ± 1.0	4.5 ± 1.0	4.0 ± 0.8	5.9 ± 1.7	10.6 ± 2.6	8.3 ± 2.1
3FL	111.3 ± 42.7	605.8 ± 456.9	224.2 ± 36.0	115.7 ± 24.6	169.3 ± 41.6	236.7 ± 71.0
Total	1980 ± 145	1969 ± 425	1282 ± 128	1044 ± 107	1141 ± 153	853 ± 247

Data shown as mean with SEM. 2′FL: 2′-fucosyllactose; LNnT: lacto-N-neotetraose ; 6′SL: 6′-sialyllactose ; 3′-SL: 3′-sialyllactose; 3FL: 3-fucosyllactose.

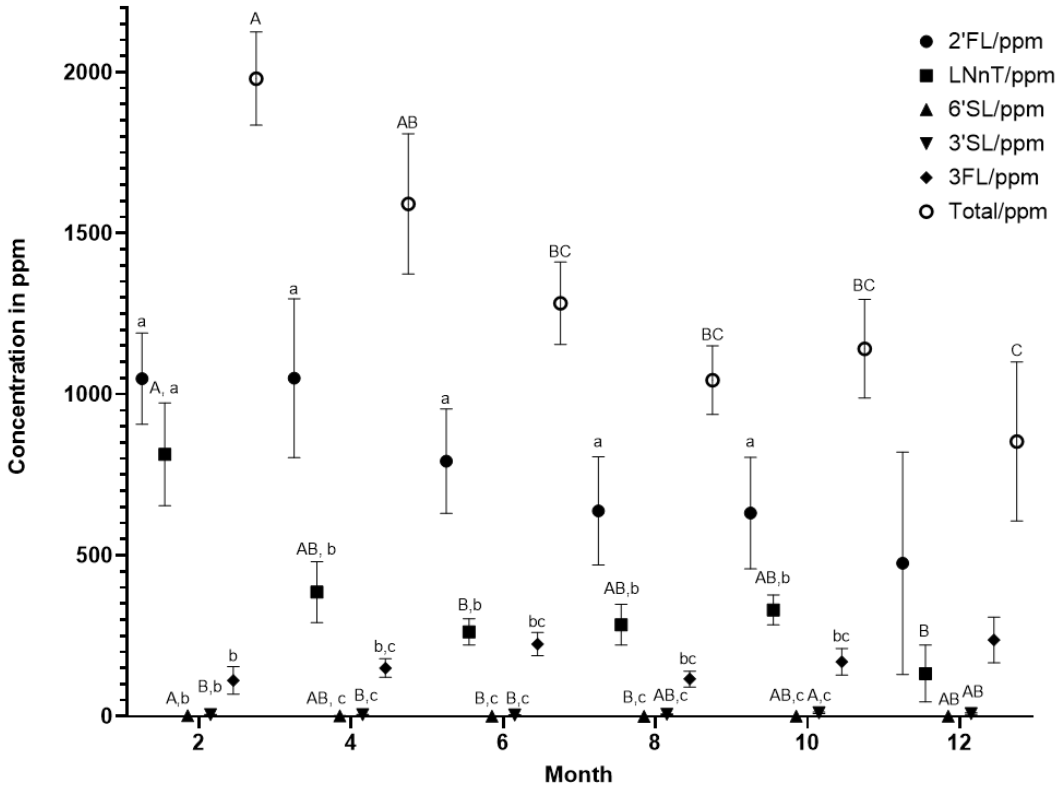


Figure 6. 1 5 most abundant HMOs throughout the lactation period.

Data shown as mean with SEM. 2′FL: 2′-fucosyllactose; LNnT: lacto-N-neotetraose ; 6′SL: 6′-sialyllactose ; 3′-SL: 3′-sialyllactose; 3FL: 3-fucosyllactose. The different capital letters (A-C) represent significant difference of same HMOs across different month. The different superscript letters (a-c) represent significant difference of difference HMO within same month.

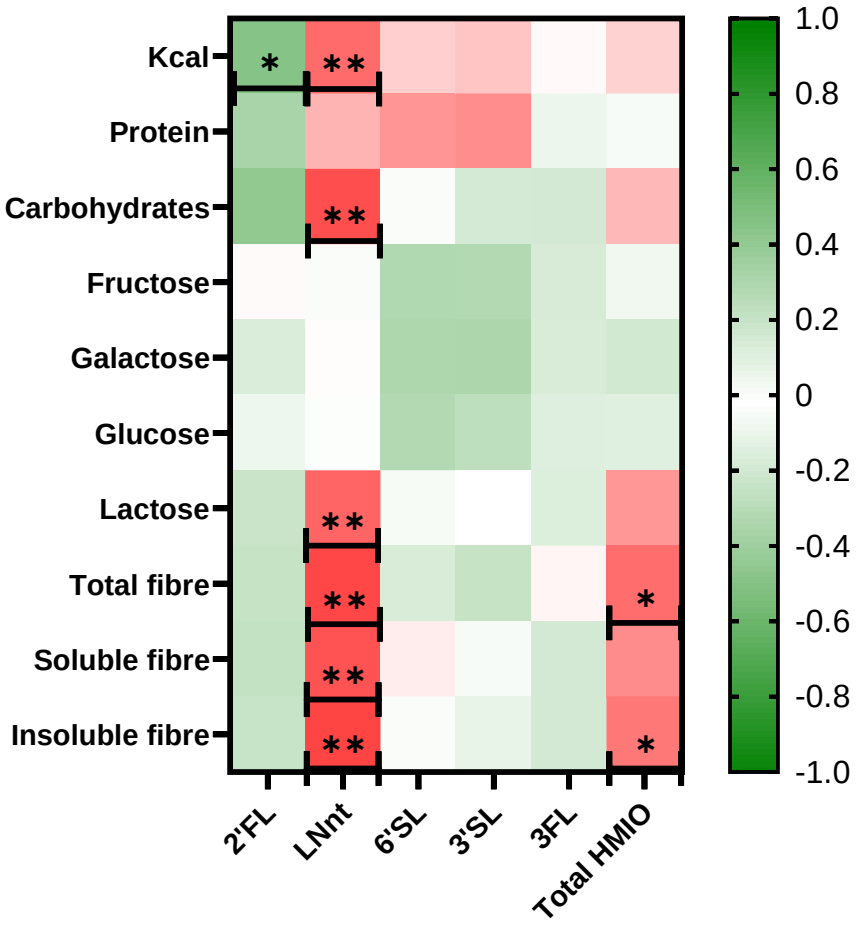


Figure 6.2 Correlation between maternal diet intake of macronutrients and dietary fiber. during lactation and individual HMO concentration.

* and ** presents a significant level of < 0.05 and < 0.01 respectively. The red color indicates a positive correlation while green color indicates a negative correlation. 2′FL: 2′-fucosyllactose; LNnT: lacto-N-neotetraose; 6′SL: 6′-sialyllactose; 3′-SL: 3′-sialyllactose; 3FL: 3-fucosyllactose.

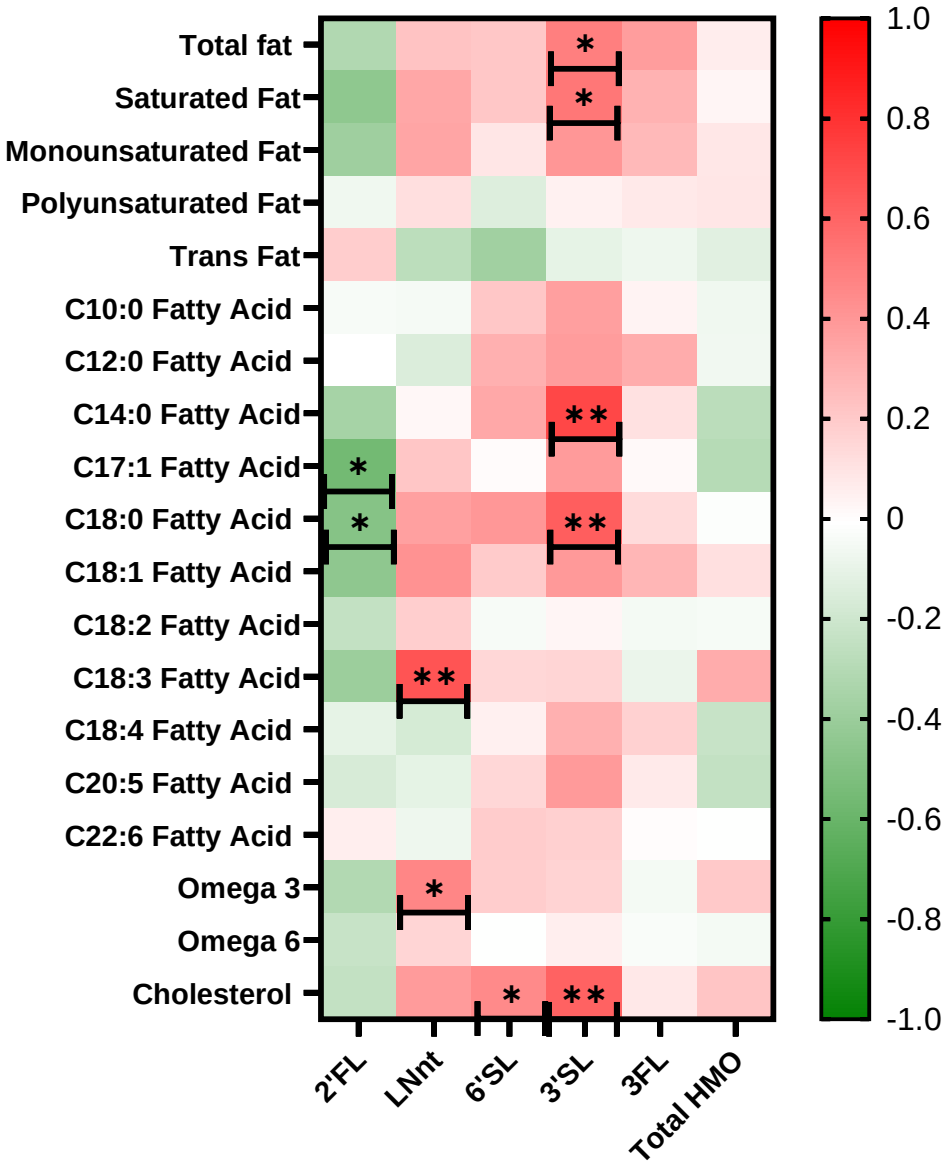


Figure 6.3 Correlation between maternal dietary fat intake during lactation and individual HMO concentration.

* and ** presents a significant level of < 0.05 and < 0.01 respectively. The red color indicates a positive correlation while green color indicates a negative correlation. 2′FL: 2′-fucosyllactose; LNnT: lacto-N-neotetraose; 6′SL: 6′-sialyllactose; 3′-SL: 3′-sialyllactose; 3FL: 3-fucosyllactose.

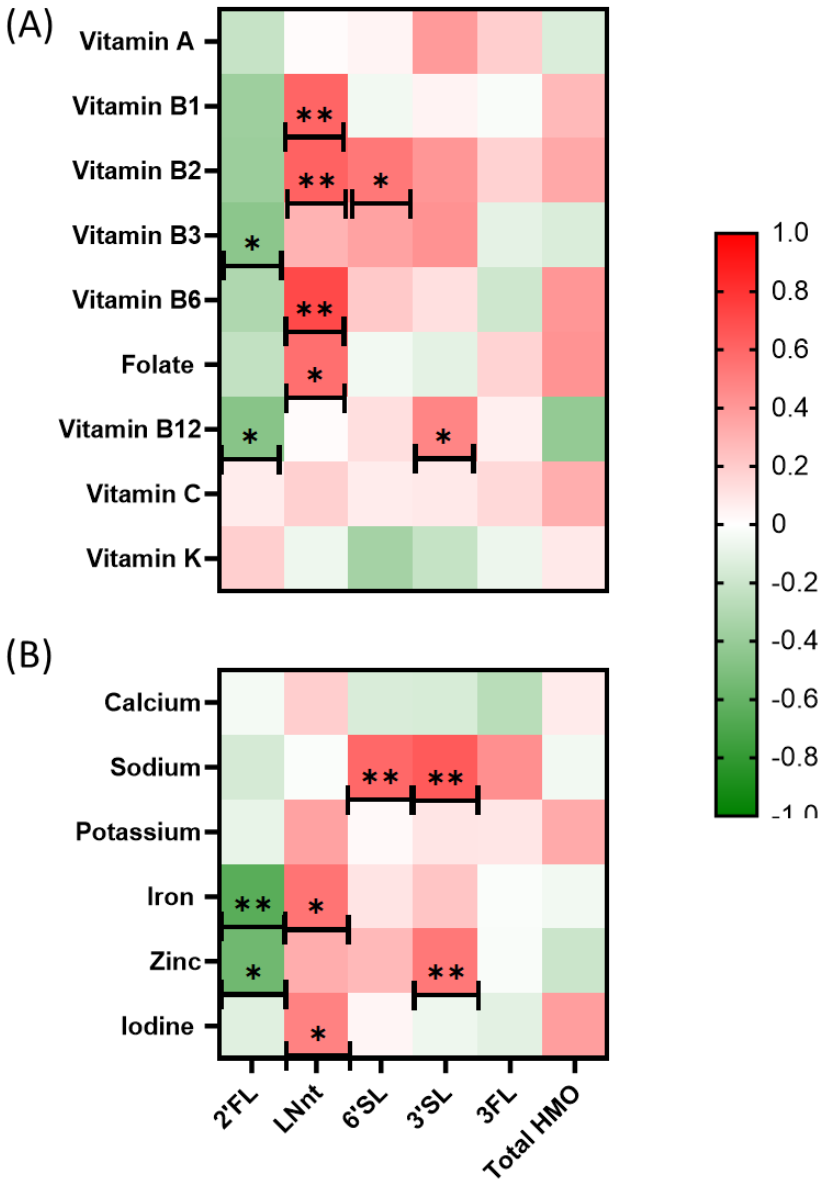


Figure 6.4 Correlation between maternal micronutrient intake during lactation and individual HMO concentration.

(A) Vitamins intake during lactation. (B) Minerals intake during lactation. * and ** presents a significant level of < 0.05 and < 0.01 respectively. The red color indicates a positive correlation while green color indicates a negative correlation. 2′FL: 2′-fucosyllactose; LNnT: lacto-N-neotetraose; 6′SL: 6′-sialyllactose; 3′-SL: 3′-sialyllactose; 3FL: 3-fucosyllactose.

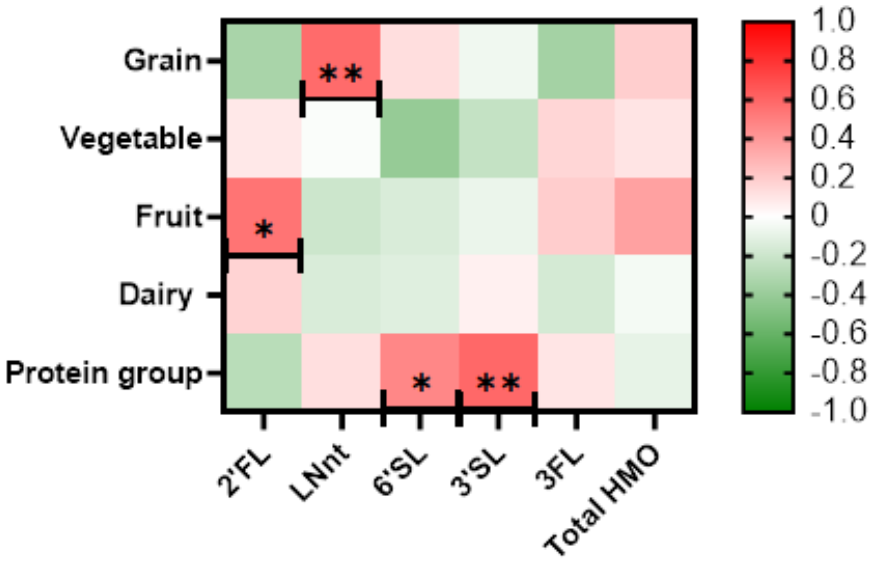
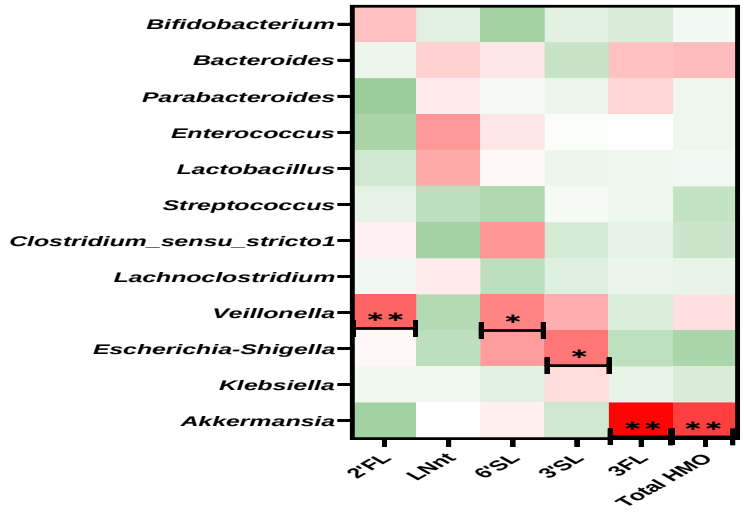


Figure 6.5 Correlation between maternal food group intake during lactation and individual HMO concentration.

* and ** presents a significant level of < 0.05 and < 0.01 respectively. The red color indicates a positive correlation while green color indicates a negative correlation. 2′FL: 2′-fucosyllactose; LNnT: lacto-N-neotetraose; 6′SL: 6′-sialyllactose; 3′-SL: 3′-sialyllactose; 3FL: 3-fucosyllactose.

(A) Pearson Correlation



(B) Spearman Correlation

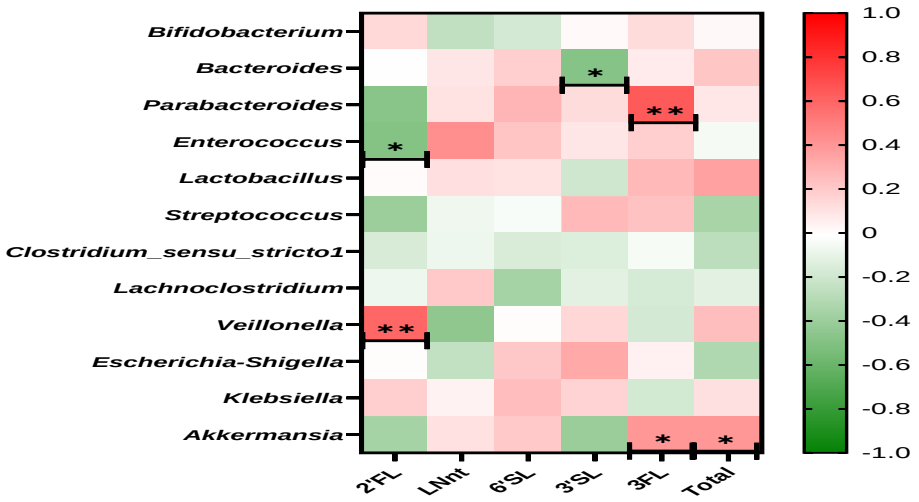


Figure 6.6 Correlation between HMOs in breast milk and infant gut microbiota at genus level.

(A) Pearson Correlation. (B) Spearman Correlation. * and ** presents a significant level of < 0.05 and < 0.01 respectively. The red color indicates a positive correlation while green color indicates a negative correlation. 2′FL: 2′-fucosyllactose; LNnT: lacto-N-neotetraose; 6′SL: 6′-sialyllactose; 3′-SL: 3′-sialyllactose; 3FL: 3-fucosyllactose.

6.4 Discussion

HMOs are the third most abundant components in breast milk, which contribute to health benefits for infants even though they cannot be digested by human digestive enzymes. In addition, the structures of HMOs are very complex, and more than 200 types of HMOs have been identified. Different sets of oligosaccharides could be detected in the breast milk of different women (Kobata, 2010). The composition would be distinctive in the milk of mothers with *FUT2* gene and *FUT3 gene* (secretor) when compared to the milk of those mothers without these two genes (non-secretors) (Kunz et al., 2017). One of the major HMO differences between secretor and non-secretor is 2′FL concentration. A research study conducted in China had used the concentration to determine whether the milk is from secretor through the concentration of 2′FL; >0.2 g/L in their milk was classified as secretor milk (Wu et al., 2020). In addition, it is suggested that other factors also could affect the HMO concentration in breast milk and one of the factors is maternal diet during lactation. Therefore, the aim of this study was to examine some major types of HMO in the mature human milk of mothers in Hong Kong, and to investigate if there are any associations with maternal nutrient intake during lactation.

2′FL was the most abundant HMO in human milk samples in this study, which was consistent with the study conducted in a cohort of lactating women in Australia (Biddulph et al., 2023) which the breast milk samples were collected form healthy Australian mothers who were 3-4 months after delivery and breastfeeding their infant. 64% of mothers were aged between 31-40, and 35% were between 20-30, which were similar to the age of our study (68% between 30-40 and 32% between 20-30). The total HMO concentration ($p<0.05$) and 2′FL reduced throughout the course of lactation.

Although it is not significant, these two findings were consistent with a review study which analyzed the HMO content in milk from day 0 to late lactation (> 90days) of other studies conducted in different countries, including China and Japan in Asia, USA, and Europe (Soyyilmaz et al., 2021). This implies that these observations are common in different races.

However, some results findings were not consistent with the one in Plows et al. (2021) which showed that 2′FL was steady throughout the lactation period while 3FL steadily increased from 1 month to 24 months. The authors raised out that the result was inconsistent with the majority of the previous studies and proposed that the reason may be due to Hispanic participants were the study target in the study. Further study is needed to understand the changes of HMO concentrations and their functions.

To our best knowledge, limited studies have been conducted to investigate the association with maternal diet during lactation and HMO concentration in human milk. In our study, we found that dietary fiber intake includes soluble and insoluble dietary fiber were positively correlated with LNnT while fruit intake was associated positively with 2′FL in human milk. The result was comparable to the one reported by Quin et al. (2020) that fruit intake was positively correlated with selected HMOs which is a source of simple sugar and dietary fiber. They also indicated that the fiber derived from fruit were positively correlated with galactose and fucose present in HMO (Biddulph et al., 2023). The correlation observed in our study between dietary fiber and HMOs in breast milk was consistent with the result showing that dietary fiber intake of mother during lactation was associated with the secretor HMO profiles (Selma-Royo et al., 2022). The association between maternal dietary intake during lactation may be due to those

monosaccharides, such as glucose, galactose and fucose, are part of the major monomers of HMO biosynthesis (Biddulph et al., 2023).

Quin et al. (2020) conducted the first study to report that HMOs were positively correlated with monounsaturated and polyunsaturated fatty acid especially some sulfonated HMOs while a opposite trend was observed in saturated fatty acid intake in lactating mothers. Our study also showed that intake of a saturated fatty acid C18:0 was negatively associated with 2′FL while intake of C18:3, an unsaturated fatty acid, and omega 3 fatty acid were positively correlated with LNnT. C18:0, Stearic acid is one the most common saturated fatty acid in our diet which can be found in animal fats. High intake of saturated fat is associated with higher risk of cardiovascular disease while C18:3, alpha linolenic acid is an essential fatty acid that human must get from diet. C18:3 can be found in plant oil such as flaxseed oil and canola oil which is an omega 3 fatty acid contributing to cardiovascular health (Calder, 2015). These two observed correlations propose that diet with healthy fat may associate with high HMO concentration of 2′FL and LNnT. However, we observed a positive correlation between intake of total saturated fat and some saturated fatty acid and 3′SL in our study. In brief, the findings of this study were insufficient to provide a conclusive summary and further research is needed to elucidate the relationship between fat intake of maternal diet and HMOs in breast milk.

The data on maternal dietary micronutrient intake and HMOs concentration in milk were also limited. Our data showed that there were positive associations between HMOs such as LNnT in milk and certain micronutrients such as vitamin B1, B2, B6,

folate, and iron. LNnT is one of the important core structures of HMOs in human milk which exist as free oligosaccharides or derivatives (Chen, 2015). For the biosynthesis of LNnT, Lacto-N-triose II (LNT II) should be first synthesized and then converted to LNnT by the help of certain enzymes such as β-galactosidases and β-1,3-Galactosyltransferase (Zhu et al., 2022). Some of the vitamin Bs are important co-factor / co-enzyme for enzyme function. It was proposed that supplement of folic acid and vitamin B_{12} during early lactation increased milk production (Preynat et al., 2009), and a study supplemented cow during early lactation with folate and vitamin B12 showed a 2.7-fold change in gene expression of beta-1,3-galactosyltransferase 2 (Gagnon et al., 2015). Although this was an animal study it gave some insights that the intake level of vitamin B, especially folate, may affect the biosynthesis of LNnT and the concentration present in human milk.

It was suggested that LNnT has a prebiotic effect, which is known as a unique prebiotic, that may promote the growth of *Bifidobacterium infantis* (Marcobal et al., 2011). It was shown that 2′FL/LNnT were able to strengthen the gut barrier function *in vitro* which maintains the integrity of the gut and bring beneficial effects on health (Natividad et al., 2022). Therefore, if maternal dietary intake during lactation can influence the HMOs concentration in breast milk, we may be able to bring extra health benefits to our next generation via altering the maternal diet during breastfeeding.

Dietary information at only one time point and small no. of sample sizes may be the limitation of this study. However, this is the first study to explore the correlation between HMO in the breast milk of Hong Kong lactating mothers and a comprehensive diet analysis. A longitudinal study with dietary information and milk collection

throughout the course of lactation to give a clearer picture on how maternal diet during lactation affects the HMOs throughout the lactation period is needed in the future.

HMOs can't be digested by human digestive enzymes but can be utilized by some beneficial bacteria in the gut of infants. The growth of these beneficial bacteria may outcompete the pathogenic ones, promoting the gut microbiota towards a more homeostatic status. At the same time, these bacteria may produce some metabolites such as short chain fatty acid which play important physiological roles including modulation of the intestinal barrier, serving as energy for enterocytes, and involving in epigenetics (Walsh et al., 2020).

In this study, the HMOs in breast milk were correlated with the infant gut microbiota. We did not observe any correlation between HMOs in breast milk and *Bifidobacterium* with statistical significance. It is suggested that *Bifidobacterium longum* subsp. *infantis* could use HMOs as a carbon and energy source efficiently and some strains of *Bifidobacteria*, yet this observation did not apply to all *Bifidobacteria* (Arzamasov et al., 2022; Walsh et al., 2020). This fact could be one of the reasons why no significant correlation was observed between genus *Bifidobacterium* in infant fecal samples and HMOs in breast milk.

In our study, *Akkermansia* in infant gut was positively correlated with total HMOs in milk which included the sum of 2′FL, LNnT, 6′SL, 3′SL and 3FL. Flores' research group has conducted a study on the utilization of different purified HMOs by different strains of *Akkermansia*. Their results showed that some strains of *Akkermansia* could growth well on media with 2′FL, 3FL, LNnT, and 6′SL as the carbon source even though the

growth yields varied across strains. The higher growth rate of these *Akkermansia* strains may partially be related to higher enzyme activities of the α-fucosidases, α-sialidases, and β-galactosidases (Luna et al., 2022). *A. muciniphila* is one of the of *Akkermansia* species found in the human gut as early as one month of age and often linked with positive effects on human health (Luna et al., 2022). One of reasons for the potential health benefits of breast milk may be due to the growth of *Akkermansia* in infant gut which can utilize some types of HMOs in breast milk efficiently.

Veillonella in infant fecal samples were found to have positive correlation with 2′FL and 6′SL in our study. The information on *Veillonella* and HMOs is limited from the literature. Geddes' group reported a study that suggested the HMOs in breast milk may affect the growth of milk microbiota. In their findings, *Veillonella* was one of the genera that was affected by the HMOs present in breast milk (Cheema et al., 2022). The association found in our study may be partially due to the microbiota presence in milk which could be passed to infant and affect their microbiota in gut.

This study tried to explore the HMOs in breast milk samples and the relationship between the HMOs with maternal diet intake during lactation and the infant gut microbiota. One of the limitations of this study is lack of information related to the genetic status of mothers that whether they were "secretor" or "non secretor". It has been reported that HMOs secretion will be different in secretors' and non-secretors' profiles. Some factors affecting HMOs concentration in breast milk are secretor dependent. Therefore, the analysis would be clearer if this piece of information is known. In addition, the use of only a few HMO standards in this study and small sample size are also some other limitations of these study. In addition, the composition of breast

milk including bioactive compound and nutrients may vary and fluctuate throughout different period in a day (Italianer et al., 2020). However, the breast milk samples in our study were collected when mothers came to our university which was either in morning session or afternoon session. Therefore the variation due to the timing of breast milk collection may exist which was also one of the limitation of our study.

Despite the above limitations, the study showed some correlation between HMOs, maternal diet during lactation and infant gut microbiota. It demonstrated that maternal diet could be a factor that we can manipulate in order to alter the HMOs in breast milk. Also, we could show that the HMOs have the possibility to change the infant gut microbiota. Nonetheless, we understand that the beneficial effects of breastfeeding are not limited to the HMOs present in the breast milk, direct transfer of microbiota from mothers to infants via human milk is also one of the proposed mechanisms. Thus, milk microbiota is also worth investigating to understand the beneficial effects of breast milk on infants. The association between maternal diet and milk microbiota is also of importance to examine.

Chapter 7 Discussion and Conclusion

7.1 Overall Discussion

Developmental programming is a worthy research area. Since many factors affect the health of the next generation, it is valuable to examine if there is any way to improve their health. It is proposed that the first 1000 days of life is a critical window for us to modify the risk factors of life-long diseases. Thus, pregnancy and lactation period are important timing for investigation.

Many stimuli or factors during pregnancy and lactation will affect the next generation's health via epigenetic mechanisms, however, not all factors are modifiable. Modifiable factors are worth exploring; one is maternal nutrition. Food is our daily necessity; humans can become healthier with a lower risk of diseases through making healthier food choices and having a balanced diet. The diet of mothers not only can influence the health status of the mother herself but also affect the health of their babies.

7.1.1 Maternal Diet in Hong Kong

The diet in Hong Kong in general, consists of inadequate intake of vegetables and fruit but high seafood and fish consumption. One of the objectives of our study is to assess the maternal diet during pregnancy and lactation in Hong Kong. In this research study, pregnant and lactation women were recruited for diet assessment by Food Frequency Questionnaire and 3-day diet record, respectively.

The diet analysis showed that the average intake of vegetables, fruits, and dairy products in pregnant and lactating women was inadequate when compared with the recommendation of MyPlate and Department of Health (HK). In general, more than

90% of the subjects did not meet the recommendation.

Regarding energy intake, the total calories reported in the FFQ was only 1357.2±80.1 kcal during the last trimester of pregnancy, while the average energy intake of breastfed and mix-fed mothers was 2341.2±142.0 and 2111.6±136.3 kcal respectively. More than 90% of the pregnant women in our study did not meet 2250kcal (Recommendation of Chinese DRI 2013), and 60.0% of mothers who exclusively breastfed their child had less than 2300kcal (Recommendation of Chinese DRI 2013) a day. Generally, most mothers during lactation could meet the carbohydrate and protein intake recommendation suggested by US DRI and Chinese DRI (more than 80%). However, more than 50% of the pregnant women did not meet the requirement. The insufficient intake of energy, including carbohydrate and fat intake, will affect the health status of mothers as well as the development of the fetus or infants.

One of the major problems of the maternal diet during pregnancy or lactation in Hong Kong is inadequate dietary fiber intake. The majority of the pregnant and lactating women had less than 50% of the recommended dietary fiber intake. Dietary fiber poses many health benefits to mothers; for example, it lowers the risk of developing cardiovascular diseases, obesity, and some gastrointestinal diseases. The intake of soluble fiber could improve insulin sensitivity. Adequate dietary fiber intake could lower the risk of constipation, gastroesophageal reflux disease, and hemorrhoids (Anderson et al., 2009). Maternal dietary fiber intake could influence the maternal gut microbiota, human milk oligosaccharides content in breast milk, and milk microbiota, which in turn affect the gut microbiota of the infants.

Inadequate intake of Vitamin A, Vitamin Bs, calcium, and iodine from the diet were the problem of maternal diet during pregnancy or lactation. However, most of the mothers had taken supplements in these two periods. Considering the most common type of supplement mothers took, most micronutrient requirements except Vitamin A and Calcium could be met. Vitamin A deficiency in lactating women may affect the content in breast milk and predispose their infants to the risk of vitamin A deficiency and increase the risk of xerophthalmia, growth retardation and mortality (Lu et al., 2022). The calcium deficiency may affect the bone health of mothers especially during breastfeeding which may trigger bone in mothers to release calcium for milk production (Bae & Kratzsch, 2018).

Another concern in the maternal diet was an excess intake of sodium during lactation. High sodium intake increases the risk of cardiovascular diseases; therefore, processed food and sauces with high sodium content should be avoided. Fresh food with minimal processing is recommended.

The self-reported dietary information from FFQ showed a relatively low energy intake from pregnant women in our study. The result may be due to underestimation of oil/fat consumption since the information of cooking method was lacking and it suggest possible limitations of using FFQ, in which recall bias exists, and the respondents seemed to underestimate their food intake. To get more accurate dietary information during pregnancy, we could ask the mothers to fill in FFQ and at the same time we collected a 3-day diet record to get more information about the dietary habits of the mothers.

7.1.2 Maternal Diet, Feeding Modes, and Gut Microbiota of Infants in Hong Kong

Maternal nutrition is one of the factors influencing the development of infant gut microbiota. The intestinal microbiota has been shown to link with human health and the infant gut microbiome plays an important role in the growth and especially the development of the immune system. The first colonization of microorganisms in gut occurs at birth and gradually becomes mature as adult-like microbiota. The disruption of early colonization may increase the risk of diseases. In addition to the diet of mother during pregnancy and lactation, feeding modes are also one of the significant factors.

Metagenomics analysis has been carried out on infant fecal samples in US and Europe to examine infant gut microbiota development and the effects of different factors on the development. However, these data are rather lacking in Hong Kong. Some studies have been conducted to study the influence of maternal diet during pregnancy on infant gut microbiota, yet the effects of maternal diet during lactation were limited. Therefore, the aims of our study are to study the gut microbiota development in Hong Kong infants and examine if maternal diet and feeding mode affect the development of the gut microbiota in infants.

7.1.2.1 Feeding Modes and Gut Microbiota of infant in Hong Kong

The alpha diversity of infant gut microbiota was lower at 2 months of age than those at older ages. The observation was in line with the result of other studies. The phyla Bacteroidetes, Firmicutes, Proteobacteria, and Actinobacteria were the dominant bacteria across the first year in the fecal samples of infants. Regarding the effects of feeding modes, early feeding pattern has been proposed to be the single and most significant factor affecting the infant gut microbiome. In our study, a higher abundance

of *Bifidobacterium* were found in the fecal samples of exclusively breastfed infants at months 2 and 4. which indicated that higher abundance of well-known good bacteria present in breastfed infants. However, this observation disappeared from the 6th month onwards when solid food introduction started. This result could be due to the introduction of solid foods, which initiated the changes in the gut microbiota and gradually reduced the profound effect of breastfeeding.

7.1.2.2 Maternal Diet during Lactation and Infant's gut microbiota

Breastfeeding provides a natural ideal nutrient source for infants and at the same time, it may affect the infant's gut microbiota development. Some mechanisms were proposed, such as hormones and antibodies, human milk oligosaccharides (HMO) and milk microbiota in the breast milk. Maternal nutritional status or dietary intake has been shown to influence the HMO content in breast milk and bacterial composition of human milk, directly or indirectly affecting the infant gut microbiota development. However, the information in these areas was scarce. Thus, correlational analyses were conducted in this study to examine if there is any association between maternal diet during lactation and the infant gut microbiota first.

The result of our study indicated that some nutrient intake during lactation in mothers correlated with the infant gut microbiota. Maternal total fiber intake was positively correlated with the family level Tannerellaceae; this group can utilize indigestible polysaccharides to produce short-chain fatty acid propionate (Polansky et al., 2015) and butyrate (Poeker et al., 2018). In addition to dietary fiber intake in mothers, certain medium chain saturated fatty acid was positively correlated with Genera *Escherichia-shigella,* while polyunsaturated fatty acid intake was positively correlated with

Klebsiella. Lipopolysaccharide is a component of the outer membrane of Gram-negative bacteria (Minihane et al., 2015) including family Enterobacteriaceae and genus *Escherichia and Klebsiella* which is linked with chronic low-grade inflammation because lipopolysaccharide activates Toll-like receptor 4 and induces inflammatory response. Chronic low-grade inflammation has been linked to higher risk of insulin resistance and Type II diabetes (Beam et al., 2021).

The result indicated that maternal diet could influence the gut microbiota in infants and may affect the health of the infants. High dietary fiber intake in mothers might bring health benefits to infants through short-chain fatty acid produced by Tannerellaceae in infants' gut. In contrast, high saturated fatty acid intake may have a higher risk of inducing chronic low-grade inflammation in infants through a higher abundance of *Escherichia and Klebsiella* in the gut of infants. Mothers may choose Omega 3 fatty acids instead of omega 6 fatty acids in the diet since no positive correlation was found between total omega 3 fatty acid intake and *Escherichia and Klebsiella*.

The sample size of this study was small; more subjects should be recruited for sample collection and analysis. In this current study, we focused more on the effects of a lactation diet. The impact of maternal diet during pregnancy and the gut microbiota of mothers could be further investigated because other research studies showed that the gut microbiota in mothers could pass to the fetus directly and affect the future development of gut microbiota. In addition, in our study, we cannot conclude if the effects are long-lasting. Our current study was one year; a more extended follow-up period may give a clear picture.

7.1.3 HMO in Breast milk and Maternal diet during lactation

Since maternal diet during lactation may affect the HMO content in breast milk and influence the infant gut microbiota. Breast milk samples were collected, and HMO content was analysed in this study. Our results showed that the HMO concentration was dynamic throughout the lactation period in this study. The most abundant HMO in the mature milk throughout the whole lactation period was 2'FL. The concentration of LNnT was the highest in the breast milk of 2nd month and reduced afterward. Total HMO concentration reduced throughout the lactation period ($p<0.05$). We also tried to correlate the maternal dietary intake during lactation with the HMOs in breast milk samples. Not many studies have been conducted to investigate the association between maternal diet and HMO concentration in human milk. Therefore, the information in this field is limited. In our study, dietary fiber intake includes soluble and insoluble dietary fiber was found to have a positive correlation with LNnT, while fruit intake was associated positively with 2′FL in human milk. Fiber derived from fruit had been shown to positively correlate with galactose and fucose present in HMOs (Quin et al., 2020). The association may be due to activated monosaccharides being the raw material of HMO biosynthesis (Biddulph et al., 2023). Therefore, the result implied that high fiber or fruit intake might induce a high level of LNnT and 2′FL in breast milk. However, the intake of fruit and dietary fiber in Hong Kong during lactation was low compared to the recommended level.

Our study also showed that intake of a saturated fatty acid C18:0 intake in mothers was negatively associated with 2′FL in breast milk while intake of C18:3, an unsaturated fatty acid, and omega 3 fatty acid were positively correlated with LNnT. Stearic acid (C18:0) is abundant in animal fat, therefore the result suggested that mothers should

replace animal fat with fat from vegetable sources or omega 3 fatty acid to increase 2′FL and LNnT content in breast milk. LNnT has a prebiotic effect which may promote the growth of *Bifidobacterium infantis* (Marcobal et al., 2011), and 2′FL/LNnT was able to strengthen gut barrier function, which maintains the intact of the gut and bring beneficial effects on health (Natividad et al., 2022).

There were some positive associations between HMO in milk, such as LNnT and certain micronutrients such as vitamin B1, B2, B6, folate. Even though the mechanism behind this is not clear, it is proposed that certain enzymes such as β-galactosidases and β-1,3-Galactosyltransferase involved in the biosynthesis of LNnT (Zhu et al., 2022) and some vitamin Bs are important coenzymes for enzyme function; therefore, Vitamin Bs intake may affect the concentration of HMO present in human milk.

HMOs in breast milk are different in mothers of "secretor" and "non-secretor" status; however, this information is lacking in our study. Future studies should involve blood collection from mothers to identify if mothers are "secretor" and "non-secretor" status for more accurate analysis.

7.1.4 HMOs in Breast Milk and Infants' Gut Microbiota

HMOs are utilized by some beneficial bacteria in gut. We have analysed the correlation between HMOs and infant gut microbiota. Not many correlations were observed except positive correlation in genus *Akkermansia, Parabacteroids and Veillonella*. *Akkermansia* in infant's gut was positively correlated with total HMOs in milk, including the sum of 2′FL, LNnT, 6′SL, 3′SL, and 3FL. There are many strains in *Akkermansia ,* one of them is *Akkermansia muciniphila* which often are linked with

positive effects on human health (Luna et al., 2022). Hence, one of reasons for the potential health benefits of breast milk may be due to the growth of *Akkermansia* in infants' gut. HMO present in breast milk may affect the growth of *Veillonella* in milk. The association found in our study may be due to the microbiota in milk, which will be passed to infants and affect their microbiota in the gut. However, we did not measure the milk microbiota in our study so we cannot confirm this proposed mechanism. It is worth investigating the microbiota present in the milk samples and finding out if there is any correlation with the bacteria found in the gut of infants.

7.2 Conclusion

In summary, infant gut microbiota development is variable and shaped by different factors, including maternal diet and early feeding modes. Exclusively breastfeeding increased the well-known beneficial genus *Bifidobacterium* and *Lactobacillus* level in infants at month 2-4. Maternal diet during lactation is associated with some HMO in breast milk and is one of the factors shaping the development of gut microbiota in infants. Nonetheless, our result showed that the maternal diet during pregnancy and lactation in Hong Kong lack vegetable, fruits, and dairy. The findings of this study could provide scientific evidence to draw the attention of public health to adopt a healthy diet during pregnancy and lactation, which promotes good quality breast milk that benefits the development of healthy gut microbiota in Hong Kong infants.

Appendices

Appendix 1 : Informed Consent Form 1

Informed Consent Form

Effectiveness of a community-based early nutrition program on promoting breastfeeding and optimizing infant growth and diet quality

You are invited to participate in a study conducted by Prof. Tam Wing-hung of the Department of Obstetrics & Gynaecology at the Chinese University of Hong Kong and Prof. Man-sau Wong of the Department of Applied Biology and Chemical Technology at the Hong Kong Polytechnic University..

What is the reason for doing this study?

There is limited data on postpartum interventions in the community that optimize maternal and infant nutrition through improving success of breastfeeding, infant growth diet quality and microbiota to enhance health in the adulthood. We hypothesized that early nutrition program could increase breastfeeding rate, improve growth status, diet quality and gut microbiota of the infants, which in turn reduce the risk of NCDs in their adulthood. This is a pilot study aiming to set up a community based early nutrition program, including breastfeeding workshops and supports, healthy lifestyle courses, parenting education, introduction of solid foods for infants, child development and cooking classes of infant foods. We will evaluate the effectiveness of this early nutrition program and determine its impacts on breastfeeding, infant growth, diet quality and infants gut microbiota, as well as the benefits to the mothers such as reducing the postpartum weight retention.

What you will do if you choose to be in this study?

After delivery (before discharge from the postnatal ward):

Our research staff will carry out physical examination (measure body weight and height) for you. Your husband will have to report his own body weight and height. We will ask your demographic and other characteristics and to complete a questionnaire on nutrition knowledge, together with a Food Frequency Questionnaire. We will measure your baby's weight, length and head circumference.

Then, you and your child will be assigned into either one of the two groups.

(i) Intervention group
All subjects will receive an early nutrition program for a period of 12 months at The Hong Kong Polytechnic University (PolyU). The workshops include talks and experience sharing sessions run by lactation consultants, nutritionists / dietitians.

(ii) Control group
Control group will receive standard health care in PolyU.

You can refer to the flowchart (appendix 2) for the tasks and measurement at different time points at the first year postpartum. The information and specimens collected may

be used in other areas of clinical research in future.

What are some of the risks and discomforts that may happen to people who are in this study?

We only carry out simple physical examination which has no risk or discomfort

What are some of the benefits that are likely to come from my being in this study?

There is no direct benefit from joining the study. However, PolyU will provide a one year eye examination at 2nd, 6th and 12th month at the Optometry Clinic free of charge. The optometrists use special techniques with the aid of sophisticated paediatric equipment to examine the visual conditions (refractive errors and ocular growth) of infants.

What are my rights as a research subject?

You do not have to take part in this study, and you have the right to refuse or withdraw at any time without jeopardizing your right to receive treatment in the hospital.

What about my confidentiality and privacy rights?

Your identity and information will not be disclosed to any irrelevant parties (NTEC-CUHK Cluster REC/IRB is one of the authorized parties to access the subjects' records related to the study for ethics review purpose). The results of this study may be presented at meetings or in publications but your identity will not be disclosed in these presentations.

If I have questions or concerns about this research study, whom can I call?

This study is coordinated by Prof. Tam Wing Hung, Department of Obstetrics & Gynaecology, the Chinese University of Hong Kong. If you have further questions you can contact research coordinators Ms. Fanny Ng Yuk Fan at 3400 8859 (Department of Applied Biology and Chemical Technology, PolyU). You are also welcome to contact NTEC-CUHK Cluster REC/IRB at 3505 3935 for ethical matters.

Consent Summary:

This study has been clearly explained to me and I have read and understood the information provided. I agree that I am enrolled in the study. I understand that I have the right to decline that I enter the study and that I have the right to withdraw from the study at any time for any reason, without jeopardizing my right to receive treatment in the hospital.

Subject's name:	_____	Subject's signature:	_____
Date:	_____		
Name of person obtaining consent:	_____	Signature of person obtaining consent:	_____
Date:	_____		

參與者知情同意書

初生時期的營養課程如何促進母乳餵哺、均衡營養與助長嬰兒發育

閣下現獲邀參加上述之研究項目。這項研究由香港中文大學 (中大) 婦產科學系教授譚永雄醫生與香港理工大學 (理大) 應用生物及化學科技學系黃文秀教授負責。

為何要進行此研究?

香港只有少量研究是關於營養對嬰兒發育的影響。我們現進行一項先導研究，這項研究估計初生時期之營養課程可促進婦女母乳餵哺的成功率 、幫助婦女產後修身，促進母嬰均衡營養和腸道健康、助長嬰兒發育，從而有助降低嬰兒在成年後患上慢性疾病的風險。我們會提供一系列的研討會與工作坊，主題包括母乳餵哺、健康生活、育兒資訊、嬰幼兒發育、為嬰幼兒引進固體食品和嬰幼兒食物烹飪法。先導計劃所收集的數據與經驗將有利我們發展進一步的研究計劃。

研究步驟是什麼?

如你同意參與此研究，你將會被分配到[干預組] 或 [對照組]。

- 住院期間

  我們將為*你和*嬰兒進行體格檢查。我們會為你們測量身高、體重和頭圍(只適用於嬰兒)。我們亦會收集*你*的家庭背景、丈夫的身高和體重等資料以及邀請*你*填寫一份關於營養知識和飲食習慣的問卷。

- 出院後

(i) 干預組

你將在理大參與一個為期 12 個月的初生時期的營養課程，包括出席由哺乳顧問、營養學家/註冊營養師主講的研討會和經驗分享小組。

(ii) 對照組

你將在理大獲得一套標準的健康教育材料。

此外, 附錄二的圖表詳細顯示了本研究的流程和需要收集的資料。是此研究收集所得的資料和樣本將來有機會供其他經審核的研究項目之用。

參加研究會否帶來什麼風險?

檢查均屬無痛與非侵入性，不會為你們造成任何痛楚或風險。

我會否因此獲益?

你並不會獲得任何酬勞。然而，香港理工大學眼科視光學診所將為所有嬰兒於出生後第 2 個月、第 6 個月及第 12 個月提供免費眼科檢查，檢查範圍包括屈光不正和眼睛健康情況等。

我有什麼權利?

參與此項研究與否純屬自願，你有權拒絕或於任何時候退出此項研究而不會影響你應有的治療。

如何保障我的個人私隱?

如你同意參與此研究，研究員會記錄你的身份、產科資料及病歷。研究結果會在醫學會議或出版刊物內發表，除 <香港中文大學–新界東醫院聯網臨床研究倫理聯席委員會>可以就研究倫理事宜查閱參與者的資料外，我們絕不透露你的身份及個人資料。

如有問題，可向誰查詢?

此項研究由中大婦產科學系教授譚永雄醫生與理大應用生物及化學科技學系黃文秀教授負責。如有疑問，請致電理大應用生物及化學科技學系副研究員吳玉芬女士 (3400 8859)。若有關於臨床研究倫理之查詢，可聯絡<香港中文大學–新界東醫院聯網臨床研究倫理聯席委員會> (3505 3935)。

同意書總結:

本人已詳閱並了解此項醫學研究的資料。本人明白參與此項研究與否純屬自願，有權拒絕或於任何時候退出此項研究而不會影響我應有的治療。

參與者姓名：_____ 參與者簽署：_____

日期: _____

解釋人員姓名：_____ 解釋人員簽署：_____

日期: _____

Appendix 2 : Background Information Questionnaire

初生時期的營養課程如何促進母乳餵哺、均衡營養與助長嬰兒發育

家庭背景問卷

參加者編號:_____
收集日期:_____

為更了解家庭環境對於嬰兒成長的影響，此問卷將需要搜集以下個人資料。所有資料絕對保密，只供給相關研究人員查閱。

本問卷共有三部分，請在合適的空格上加上 "✓"號，或請扼要填寫數字或文字。

一、家庭基本資料

1. 請問現在是否住在沙田區？	□ 是	□ 不是 (請註明居住區域 _____)			
2. 現在的居住面積 (實用面積)	□ 200 呎以下	□ 200-400 呎	□ 400-600 呎	□ 600-800 呎	□ 800 呎以上
3. 一同居住的家庭成員數目 (包括嬰兒)	□ 2 人	□ 3 人	□ 4 人	□ 5 人	□ 6 人或以上
4. 與嬰兒同住的家庭成員有沒有吸煙習慣？	□ 有	□ 沒有			
5. 嬰兒父母婚姻狀況	□ 已婚	□ 離婚	□ 分居		
6. 每月家庭總收入 (港幣)	□ <20000	□ 20000-40000	□ 40000-60000	□ 60000-80000	□ 80000 或以上

7. 曾經或現在患上疾病 (可選多於一項)：

(A) 我沒有患有任何疾病　(B)糖尿病　(C) 高血壓　(D) 高血脂

(E) 動脈硬化　(F) 中風　(G) 癌症　(H) 其他 (請註明)

嬰兒母親：____

_____

_____

嬰兒父親：_____

_____

嬰兒其他家族成員：_____

_____

二、嬰兒父、母親基本資料

<table>
<tr><th></th><th colspan="2">母親</th><th colspan="2">父親</th></tr>
<tr><td>姓名 (中文或英文)</td><td colspan="2"></td><td colspan="2"></td></tr>
<tr><td>年齡</td><td colspan="2"></td><td colspan="2"></td></tr>
<tr><td>職業</td><td colspan="2"></td><td colspan="2"></td></tr>
<tr><td>身高</td><td colspan="2">厘米 (cm)</td><td colspan="2">厘米 (cm)</td></tr>
<tr><td>體重</td><td colspan="2">公斤 (kg)</td><td colspan="2">公斤 (kg)</td></tr>
<tr><td>懷孕周數</td><td colspan="2">周</td><td colspan="2" rowspan="3">不適用</td></tr>
<tr><td>懷孕前體重</td><td colspan="2">公斤 (kg)</td></tr>
<tr><td>懷孕後體重 (生產前最後一次量度的體重)</td><td colspan="2">公斤 (kg)</td></tr>
<tr><td>教育程度</td><td colspan="2">□ 小學或以下
□ 中學
□ 大專
□ 大學或以上
□ 其他 (請註明 _____)</td><td colspan="2">□ 小學或以下
□ 中學
□ 大專
□ 大學或以上
□ 其他 (請註明 _____)</td></tr>
<tr><td>吸煙習慣</td><td colspan="2">□ 沒有吸煙習慣
□ 曾有吸煙習慣，但已戒煙
□ 現有吸煙習慣</td><td colspan="2">□ 沒有吸煙習慣
□ 曾有吸煙習慣，但已戒煙
□ 現有吸煙習慣</td></tr>
<tr><td rowspan="2">飲酒習慣</td><td>□ 有</td><td>如有，飲酒密度為：
□ 每星期少於 1 次
□ 每星期 1-2 次
□ 每星期 3-4 次
□ 每星期 5 次以上</td><td>□ 有</td><td>如有，飲酒密度為：
□ 每星期少於 1 次
□ 每星期 1-2 次
□ 每星期 3-4 次
□ 每星期 5 次以上</td></tr>
<tr><td colspan="2">□ 沒有</td><td colspan="2">□ 沒有</td></tr>
<tr><td rowspan="2">運動習慣 (每次 30 分鐘以上)</td><td>□ 有</td><td>如有，運動密度為：
□ 每星期少於 1 次
□ 每星期 1-2 次
□ 每星期 3-4 次
□ 每星期 5 次以上</td><td>□ 有</td><td>如有，運動密度為：
□ 每星期少於 1 次
□ 每星期 1-2 次
□ 每星期 3-4 次
□ 每星期 5 次以上</td></tr>
<tr><td colspan="2">□ 沒有</td><td colspan="2">□ 沒有</td></tr>
</table>

三、嬰兒基本資料

性別		□ 男		□ 女	
出生日期		年	月	日	
血型		□ A 型	□ B 型	□ AB 型	□ O 型
嬰兒第一口進食的是		□ 母乳		□ 配方奶粉	
體重	1	公斤 (kg)	量度日期	/ /	
	2	公斤 (kg)	量度日期	/ /	
	3	公斤 (kg)	量度日期	/ /	
身長	1	厘米 (cm)	量度日期	/ /	
	2	厘米 (cm)	量度日期	/ /	
	3	厘米 (cm)	量度日期	/ /	
頭圍	1	厘米 (cm)	量度日期	/ /	
	2	厘米 (cm)	量度日期	/ /	
	3	厘米 (cm)	量度日期	/ /	

此問卷已完成，謝謝！

Appendix 3 : Food Frequency Questionnaire Form

第二部份: 母親飲食問卷調查

參加者編號:_____

收集日期:_____

研究人員:_____

第二部份: 母親飲食問卷調查

填寫須知：這部分了解你過去三個月的飲食情況，如果你吃過後面表中所列食物，請在食用的次數空格"✓"，並填寫每次食用的份量。如果沒有吃過，請在從未的空格 "✓"

食物種類 Type of Food	過去三個月的次數 How Often Within the Past Three Months?									每次有多少 How much each time?	參考份量 Reference Portion
蔬菜類	從未 Never	一月少於一次 <1 Time per Month	一月一次 Once per Month	一月二至三次 2 – 3 Times per Month	一星期一次 Once per Week	一星期二次 Twice per Week	一星期三至四次 3 – 4 Times per Week	一星期五至六次 5 – 6 Times per Week	每日 Every day		
深綠色菜											
菜心 Choy Sum										碗 bowls	1 bowl = 150g
小白菜 Small Chinese cabbage										碗 bowls	1 bowl = 150g
油麥菜 Leaf lettuce										碗 bowls	1 bowl = 150g
生菜 Lettuce										碗 bowls	1 bowl = 150g
通菜 Water Spinach										碗 bowls	1 bowl = 150g
莧菜 Chinese Spinach										碗 bowls	1 bowl = 150g
芥蘭 Chinese Kale										碗 bowls	1 bowl = 150g
西蘭花 Broccoli										碗 bowls	1 bowl = 150g
菠菜 Spinach										碗 bowls	1 bowl = 150g
豆苗 Pea Shoots										碗 bowls	1 bowl = 150g
韭菜 Chinese Chives										碗 bowls	1 bowl = 150g
洋蔥 Onion										碗 bowls	1 bowl = 150g

蘆筍 Asparagus										碗 bowls	1 bowl = 150g
蕃薯苗 Sweet Potato-green leaves										碗 bowls	1 bowl = 150g
油麥菜 Leaf lettuce										碗 bowls	1 bowl = 150g
西洋菜 Watercress										碗 bowls	1 bowl = 150g
其他 Others										碗 bowls	1 bowl = 150g
淺綠色菜/白色菜											
娃娃菜 Baby Cabbage										碗 bowls	1 bowl = 150g
西芹 Celery										碗 bowls	1 bowl = 150g
唐生菜 Chinese lettuce										碗 bowls	1 bowl = 150g
椰菜 Cabbage										碗 bowls	1 bowl = 150g
椰菜花 Cauliflower										碗 bowls	1 bowl = 150g
其他 Others										碗 bowls	1 bowl = 150g

食物種類 Type of Food	過去三個月的次數 How Often Within the Past Three Months?									每次有多少 How much each time?	參考份量 Reference Portion
	從未 Never	一月少於一次 <1 Time per Month	一月一次 Once per Month	一月二至三次 2 – 3 Times per Month	一星期一次 Once per Week	一星期二次 Twice per Week	一星期三至四次 3 – 4 Times per Week	一星期五至六次 5 – 6 Times per Week	每日 Every day		
瓜果類蔬菜 Fruit vegetable											
冬瓜 Waxgourd										碗 bowls	1 bowl = 150g
節瓜 Wax gourd										碗 bowls	1 bowl = 150g
青瓜 Cucumber										碗 bowls	1 bowl = 150g
絲瓜 Sponge gourd										碗 bowls	1 bowl = 150g
茄子 Eggplants										碗 bowls	1 bowl = 150g
苦瓜 Bitter melon										碗 bowls	1 bowl = 150g
南瓜 Pumpkin										碗 bowls	1 bowl = 150g
蕃茄 Tomatoes										碗 Bowls	1 bowl = 150g
燈籠椒 Peppers										碗 bowls	1 bowl = 150g
紅蘿蔔 Carrot										碗 bowls	1 bowl = 150g
其他										碗 bowls	1 bowl = 150g

食物種類 Type of Food	過去三個月的次數 How Often Within the Past Three Months?									每次有多少 How much each time?	參考份量 Reference Portion
	從未 Never	一月少於一次 <1 Time per Month	一月一次 Once per Month	一月二至三次 2 – 3 Times per Month	一星期一次 Once per Week	一星期二次 Twice per Week	一星期三至四次 3 – 4 Times per Week	一星期五至六次 5 – 6 Times per Week	每日 Every day		
根莖類 Root, tuber, bulb vegetable											
馬鈴薯 Potato										碗 bowls	1 bowl = 150g
番薯 Sweet potato										碗 bowls	1 bowl = 150g
芋頭 Taro										碗 bowls	1 bowl = 150g
馬蹄 Water chestnut										碗 bowls	1 bowl = 150g
甘荀 Carrot										碗 bowls	1 bowl = 150g
白蘿白 Radish										碗 bowls	1 bowl = 150g
其他 others										碗 bowls	1 bowl = 150g
菇菌類/藻類 Mushroom and Algae											
木耳 Jew's Ear										碟 plates	1 plate=100g (wet weight)
銀/雪耳 White Fungus										碟 plates	1 plate=100g (wet weight)
黃耳 Golden Tremell										碟 plates	1 plate=100g (wet weight)
香菇 Dried mushroom										碟 plates	1 plate=100g (wet weight)
杏鮑菇 King oyster Mushroom										碟 plates	1 plate=100g (wet weight)
金針菇 Winter mushroom										碟 plates	1 plate=100g (wet weight)
紅菇 Vinous Russula										碟 plates	1 plate=100g (wet weight)
昆布 Seaweed										碟 plates	1 plate=100g (wet weight)
海帶 Sea-Tangle										碟 plates	1 plate=100g (wet weight)
海苔 Sedge										碟 plates	1 plate=100g (wet weight)
其他 Other										碟 plates	1 plate=100g (wet weight)

食物種類 Type of Food	過去三個月的次數 How Often Within the Past Three Months?									每次有多少 How much each time?	參考份量 Reference Portion
	從未 Never	一月少於一次 <1 Time per Month	一月一次 Once per Month	一月二至三次 2 – 3 Times per Month	一星期一次 Once per Week	一星期二次 Twice per Week	一星期三至四次 3 – 4 Times per Week	一星期五至六次 5 – 6 Times per Week	每日 Every day		
水果類 Fruits											
橙，西柚等 Orange, grapefruit,										個 Piece	1piece=150g
蘋果，梨，等 Apple, Pear,										個 Piece	1piece=150g
香蕉，大蕉等 Bananas, Plantains										個 Piece	1piece=150g
提子，龍眼等 Grape, Longyan										碗 bowls	1 bowl = 150g
藍莓 Blueberry										碗 bowls	1 bowl = 150g
芒果，柿子 Mango, persimmon										個 Piece	1piece=150g
奇異果 Kiwi										個 Piece	1piece=150g
車里茄 Cherry										碗 bowls	1 bowl = 150g
士多啤梨 Strawberry										碗 bowls	1 bowl = 150g
西瓜，哈密瓜等 Watermelon, Cantaloupe										個 Piece	1piece=150g
其他 Other											
豆類以及其製品 Legumes and legumes products											
鮮黃豆 Fresh Soybean										份 Servings	1 serving=50g
硬豆腐 Tofu, hard										份 Servings	1 serving=50g
布包豆腐 Tofu,soft										磚 Cubes	1cube=300g
腐竹 Dried Tofu Sheets										份 Servings	1 serving=50g

油炸豆腐 Deep Fried Tofu										份 Servings	1 serving=50g
腐皮 Tofu Skin										份 Servings	1 serving=50g
豆漿 Soy Milk										杯/盒 Cups/packs	1cup=250ml 1pack=236ml
紅豆 Red beans										份 Servings	1 serving=50g
紅腰豆 Red Kidney bean										份 Servings	1 serving=50g
綠豆 Mung Beans										份 Servings	1 serving=50g
其他 Others											

食物種類 Type of Food	過去三個月的次數 How Often Within the Past Three Months?									每次有多少 How much each time?	參考份量 Reference Portion
	從未 Never	一月少於一次 <1 Time per Month	一月一次 Once per Month	一月二至三次 2 – 3 Times per Month	一星期一次 Once per Week	一星期二次 Twice per Week	一星期三至四次 3 – 4 Times per Week	一星期五至六次 5 – 6 Times per Week	每日 Every day		
肉類											
半肥瘦豬肉 Half fat pork										安士 oz	1oz=28.5g
全瘦豬肉 Whole lean pork										安士 oz	1oz=28.5g
叉燒，燒肉 BBQ/Roasted pork										安士 oz	1oz=28.5g
豬腳，豬皮，豬扒 Pork shank, Pigskin, Pork chop										安士 oz	1oz=28.5g
牛肉(牛腩,牛丸,牛扒等) Beef, Brisket, beef balls										安士 oz	1oz=28.5g
腌制肉類如,香腸,火腿,牛餐肉等 Processed meat, sausage, ham										安士 oz	1oz=28.5g
雞 Chicken meat										安士 oz	1oz=28.5g
鴨 Duck meat										安士 oz	1oz=28.5g
鵝 Goose										安士 oz	1oz=28.5g
其他 Others											

食物種類 Type of Food	過去三個月的次數 How Often Within the Past Three Months?									每次有多少 How much each time?	參考份量 Reference Portion
	從未 Never	一月少於一次 <1 Time per Month	一月一次 Once per Month	一月二至三次 2 – 3 Times per Month	一星期一次 Once per Week	一星期二次 Twice per Week	一星期三至四次 3 – 4 Times per Week	一星期五至六次 5 – 6 Times per Week	每日 Every day		
五穀以及雜類 Grains											
糙米 Brown Rice										碗 bowls	1 bowl = 200g
糯米 Glutinous Rice										碗 bowls	1 bowl = 200g
白米 White Rice										碗 bowls	1 bowl = 200g
燕麥片 Oats										份 Servings	1 serving=50g
河粉 Flat rice noodle										碗 bowls	1 bowl = 200g
粟米片 Corn Flakes										份 Servings	1 serving=25g
意粉 Pasta										碗 bowls	1 bowl = 200g
米粉 Rice noodles										碗 bowls	1 bowl = 200g
通心粉 Macaroni										碗 bowls	1 bowl = 200g
濑粉 Lai Fen										碗 bowls	1 bowl = 200g
粉絲 Mungbean Noodle										碗 bowls	1 bowl = 200g
叉燒包，蓮蓉包等 BBQ pork buns										個 bun	1slice=25g 1bun=40g
白麵包 White bread										份 Servings	1 serving=50g
其他 Others											

食物種類 Type of Food	過去三個月的次數 How Often Within the Past Three Months?									每次有多少 How much each time?	參考份量 Reference Portion
	從未 Never	一月少於一次 <1 Time per Month	一月一次 Once per Month	一月二至三次 2 – 3 Times per Month	一星期一次 Once per Week	一星期二次 Twice per Week	一星期三至四次 3 – 4 Times per Week	一星期五至六次 5 – 6 Times per Week	每日 Every day		
淡水魚 Fresh Water Fishes											
鯪魚 Grass Fish										份 servings	1 serving = 200g
鯉魚 Mud Carp										份 servings	1 serving = 100g
鰱魚 (大魚) Silver carp (Big Head Fish)										份 servings	1 serving = 100g
生魚 Snake Head										份 servings	10 slices = 50g
鯽魚 Crucian carp										份 servings	1 serving = 100g
桂花魚 Mandarin fish										份 servings	1 serving = 100g
金山鯛、非洲鯛 Tilapia, Nile tilapia										份 servings	1 serving = 100g
加州鱸、大口鱸 Large mouth bass, Largemouth black bass										份 servings	1 serving = 100g
寶石魚 Jade Perch										份 servings	1 serving = 100g
其他 Others											
咸水魚 Sea Water Fishes											
烏頭 Grey mullet										份 servings	1 serving = 200g
鰻魚 Eel										份 servings	10 slices = 50g 1 thick slice =30g
鱸魚 Bass/Perch										份 servings	1 serving = 100g
紅衫魚 Golden Thread										份 servings	1 serving = 100g

牙帶魚 (斬件) Ribbon Fish										份 servings	1 serving = 100g 1 thick slice =30g
三文魚 Salmon										件 pieces	1 serving = 100g
九肚魚 Bombay duck										份 servings	1 serving = 100g
吞拿魚 Tuna Fish										罐 cans	1 can = 90g
盲鰽 Barramundi										份 servings	1 serving = 100g
石斑 Grouper										份 servings	1 serving = 100g
魚立魚 Seabream										份 servings	1 serving = 100g
沙丁魚、沙甸魚 Sardine and pilchard										份 servings	1 serving = 100g
大眼鱸(Big-eye perch, Red bigeye, Bulls-eye perch										份 servings	1 serving = 100g
紅鮸 Snappers										份 servings	1 serving = 100g
粗鱗撻沙、撻沙 Largescale tonguesole										份 servings	1 serving = 100g
帶仔 (斑腩) Black bonito, cobia										份 servings	1 serving = 100g
黃花魚, 白魚或 Croaker										份 servings	1 serving = 100g
蘇眉 Humphead wrasse										份 servings	1 serving = 100g
馬友 Fourfinger threadfin, Blind tasselfish										份 servings	1 serving = 100g
泥鯪 Rabbitfish										份 servings	1 serving = 100g
鯖魚 Mackerel										份 servings	1 serving = 100g
其他 Others										份 servings	1 serving = 100g

食物種類 Type of Food	過去三個月的次數 How Often Within the Past Three Months?									每次有多少 How much each time?	參考份量 Reference Portion
	從未 Never	一月少於一次 <1 Time per Month	一月一次 Once per Month	一月二至三次 2 – 3 Times per Month	一星期一次 Once per Week	一星期二次 Twice per Week	一星期三至四次 3 – 4 Times per Week	一星期五至六次 5 – 6 Times per Week	每日 Every day		
海產類 Seafood											
魷魚 Squid										份 servings	7 slices = 50g
生蠔 Oysters										份 servings	6 pieces = 50g
蠔豉 Dried Oysters										份 servings	10 pieces = 50g
蝦 Prawns										份 servings	2 pieces = 25g
蟹 Crabs										隻 pieces	1 piece = 100g
帶子/瑤柱 Scallops/Dried Scallops										份 servings	3 pieces = 20g
海參 (乾) Sea Cucumbers										份 servings	1 serving = 5g
魚丸/魚腐 Fish Balls										份 servings	5 pieces = 100g
魚片 Fish Cakes										份 servings	4 slices = 50g
墨魚 Cuttlefish										份 servings	7 slices = 50g
鯪魚球 Mud Carp Fish Balls										份 servings	2 pieces = 50g
罐頭沙丁魚 Canned Sardines										件 pieces	1 piece = 50g
罐頭豆豉鯪魚 Fried Dace with Black Bean Sauce										件 pieces	1piece = 50g
咸魚 Salted Preserved Fish										片 slices	1 slice = 5g
海蜇 Jelly Fish										份 servings	1 serving = 50g
其他 Others											

食物種類 Type of Food	過去三個月的次數 How Often Within the Past Three Months?									每次有多少 How much each time?	參考份量 Reference Portion
	從未 Never	一月少於一次 <1 Time per Month	一月一次 Once per Month	一月二至三次 2 – 3 Times per Month	一星期一次 Once per Week	一星期二次 Twice per Week	一星期三至四次 3 – 4 Times per Week	一星期五至六次 5 – 6 Times per Week	每日 Every day		
蛋類 Eggs											
煲熟雞蛋 Hard Boiled Eggs										隻 pieces	1 piece = 50g
煎雞蛋 Pan Fried Eggs										隻 pieces	1 piece = 50g
炒蛋 Stir Fried Eggs										隻 pieces	1 piece = 50g
奄列 Omelette										隻 pieces	1 piece = 50g
蛋白 Egg White										隻 pieces	1 piece = 35g
蛋黃 Egg Yolk										隻 pieces	1 piece = 15g
皮蛋 Century Eggs										隻 pieces	1 piece = 50g
咸蛋 Salted Duck Eggs										隻 pieces	1 piece = 50g
鵪鶉蛋 Quail Eggs										隻 pieces	1 piece = 10
其他 Others											

食物種類 Type of Food	過去三個月的次數 How Often Within the Past Three Months?									每次有多少 How much each time?	參考份量 Reference Portion
	從未 Never	一月少於一次 <1 Time per Month	一月一次 Once per Month	一月二至三次 2 – 3 Times per Month	一星期一次 Once per Week	一星期二次 Twice per Week	一星期三至四次 3 – 4 Times per Week	一星期五至六次 5 – 6 Times per Week	每日 Every day		
奶類及飲料 Dairy Products & Beverages											
全脂牛奶 Whole Milk										杯/盒 Cups/packs	1 cup = 250ml 1 pack = 236ml
低脂牛奶 Low-fat Milk										杯/盒 Cups/packs	1 cup = 250ml 1 pack = 236ml
脫脂奶 Skimmed Milk										杯/盒 Cups/packs	1 cup = 250ml 1 pack = 236ml
朱古力奶 Chocolate Milk										杯/盒 Cups/packs	1 cup = 250ml 1 pack = 236ml
全脂奶粉 Whole Milk Powder										湯匙 tablespoons	1 Tablespoon = 7g
低脂奶粉 Low-fat Milk Powder										湯匙 tablespoons	1 Tablespoon = 7g
脫脂奶粉 Skimmed Milk Powder										湯匙 tablespoons	1 Tablespoon = 7g
煉奶 Condensed Milk										湯匙 tablespoons	1 Tablespoon = 20g
花奶 Evaporated Milk										湯匙 tablespoons	1 Tablespoon = 15g
椰子汁 Coconut Juice										杯 cups	1 cup = 250ml
維他奶 Vitasoy										盒 packs	1 pack = 250ml
豆漿 Soy Milk										杯/盒 Cups/packs	1 cup = 250ml 1 pack = 236ml
可樂 Coke										杯/罐 Cups/cans	1 cup = 250ml 1 can = 330ml
七喜 Seven Up										杯/罐 Cups/cans	1 cup = 250ml 1 can = 330ml
益力多 Yakult										杯/罐 Cups/cans	1 cup = 250ml 1 can = 330ml
咖啡 Coffee										杯 cups	1 cup = 250ml
奶茶 Milk Tea										杯 cups	1 cup = 250ml
乳酪 Yogurt										杯 cups	1 cup = 150

Appendix 4 : 3-day Diet Record Form

三日媽媽飲食記錄及一日 BB 飲食記錄

參加者編號: _EPS_____

填寫飲食記錄指引

1. 請參考例子填寫三日(2019 年____月____日至____月____日)自己的飲食記錄, 以及一日 BB(2019 年____月____日) 的飲食記錄。
2. 請不要因為填寫此飲食及活動記錄而改變你日常的飲食/活動習慣。
3. 請緊記寫下你進食或飲用過的每一樣食物, 包括水! 亦請記下你在家以外進食過的零食及任何食物。
4. 可以的話請記下食物的**重量/份量**。如果那是一件預先包裝好的食物你可以記下標籤上的重量。如果難以做到, 你可以日常的簡單方式記低份量, 如 2 茶匙糖, 2 湯匙蔬菜, 一碗飯等。
5. 如有食物的**生產商名稱或牌子**, 請在「食物或飲品種類」一欄一併填寫。
6. 請填寫煮食方法, 例如**煎, 蒸, 炸或煮**等。
7. 請將任何自製食物的食譜簡單地記錄一下。
8. 請你簡單記下日常活動及運動情況, 以及睡眠時間。

成人進食記錄 (例子)

記錄日期： 2019 年 1 月 3 日 (星期四)

時間 Time	地點 Place	食物/飲品名稱 Food/Drink Consumed	份量 Amount
上午 7:10	屋企	白方飽 (連皮) (無搽牛油)	1 片
		煎雞蛋	1 隻
		XX 牌低脂奶	1 盒
上午 10:00	小食店	XX 牌芝士餅	1 包
		棉花糖	2 粒
中午 12:30	茶餐廳	鱔魚雞粒鳳爪飯	1 盒
		➢ 鳳爪	2 隻
		➢ 鱔魚雞粒	4 湯匙
		➢ 白飯	1 碗
下午 4:30	屋企	可樂	1 支
		朱古力糖	3 粒
晚上 7:30	屋企	白飯	1 碗
		炒牛肉	1 兩
		炝菜心	3 條
		炆節瓜，豆腐卜	2 件
		燒肉	3 件
		冬瓜瘦肉湯	1 碗
		➢ 瘦肉 (湯渣) (無食冬瓜)	2 片
晚上 8:30		橙	半個
晚上 9:00		士多啤梨味雪糕	1 杯

整日喝了幾多清水：約 6 (杯) / 毫升　　營養補充劑 (如維他命丸/鈣片) (如有)： 無

屋企用什麼油煮食： 芥花籽油

煮食鹽的用量 (鹽, 豉油, 蠔油): ☐淡味 ☑ 普通 ☐ 咸

日常活動及運動: 散步半 小時

睡眠時間: 晚上 11:00 到 早上 7:00

三日媽媽飲食記錄

進食及活動記錄表

請記錄進食之所有食物，包括早餐、午餐、晚餐、湯水、飲品、零食及消夜。請以碗、杯、湯匙、茶匙、大/小塊、大/小件或尺吋大小等，以作記錄之單位。

記錄日期：_____年_____月_____日 (星期_____)

				(不用填寫)	
時間	地點	食物/飲品名稱	份量	編號	三天份量

整日喝了幾多清水：約_____ 杯 / 毫升　　營養補充劑 (如維他命丸/鈣片) (如有)：_____

屋企用什麼油煮食：_____

煮食鹽的用量 (鹽, 豉油, 蠔油): ☐淡味 ☐ 普通 ☐ 咸

日常活動及運動: _____　　睡眠時間: _____

Appendix 5 : Informed Consent Form 2

Informed Consent Form

The association between maternal diet, early feeding practices and infant gut microbiota

You are invited to participate in study conducted by Prof. Man-sau Wong and Dr. Jiachi Chiou of the Department of Applied Biology and Chemical Technology at the Hong Kong Polytechnic University. Lilian Yeung is the project coordinator and she is willing to explain the project details to you either in Chinese or English.

1. **Purpose of the Study:**

Microbes are suggested to have a significant role on metabolic functions and immune systems. In human, there are trillions of microbiome and high density of them are found in gut. Gut microbiota in newborn affect its gut health directly and future development. Breast milk is the first nutrient source for the infant which is one of the factors modifying the gut microbiota in infants. Breast milk content is suggested to be affected by maternal diet. This pilot study is developed specifically to understand the relationship between gut microbiota of purely breastfed infant, breast milk content and maternal diet.

2. **Method of Investigation:**

The proposed project is a pilot study which subjects will be 35 local healthy infants (vaginal delivery and purely breastfed aged 2 – 4 months) from health women (18-40 years old). We collect food consumption habit from these women for dietary analysis. We also collect the breast milk sample and fecal samples from the infants (>1.5 g) in healthy condition within a week. The bacteria population will be analysed by metagenomic DNA extraction and sequencing. Comparison of bacteria population within different individual infant and breast milk content will be performed.

3. **Approach:**

Subject recruited will attend breastfeeding workshops. The pilot study will take place in the same day of breastfeeding workshop for pregnant or lactating women in PolyU. The research staff will screen participants attending the workshop, check for eligible subjects, explain study details and invite them to join the study. Informed written consent will be obtained from all subjects. The data and sample collection will be carried out on the same day of the workshop. The infants will be asked to change a diaper before the start of the workshop and the fecal samples will be collected and kept in 4^{o}C refrigerator once it is ready by the research staff. A private room will be setup for mothers to pump breast milk and a questionnaire on diet history will be conducted. On the day before the study, the research staff will contact the subject again to remind her for the interview and sample collection. We will code subject's samples so that subject's name will not be shown on the tubes (only the research team will be able to break the code) and that subject's information is kept confidential.

4. **Outcome, Relevance, Significance and Value of the Study:**

Gut microbiota is a new direction of research field. It is highly associated with health prevention, however, not much data on infant microbiota in Hong Kong can be found. By analyzing the gut microbiota and breast milk content, we may identify an association between the nutrient source and gut microbiota in infants. Diet intake of lactating

mother may be one of the factors affecting the microbiota or nutrient content of human milk. Therefore, if there is any association found from dietary record of mothers, modification of mothers' diet especially obese mothers may be a strategy to lower the risk of diseases risk in later life of their children.

5. What you will be asked to do if you volunteer to take part in this study:

A. Sign the informed consent form
B. Fill in Food frequency questionnaire on the study day
C. Provide 50 ml of breast-milk with breastfeeding
D. Provide fecal samples of infant (> 1.5 g) on the study day.
E. Provide anthropometric data (weight and height)

IT IS IMPORTANT THAT YOU FOLLOW YOUR USUAL DAILY DIETARY INTAKE DURING THE WHOLE STUDY.

6. What will we do with the breast milk, fecal samples and diet history?

We will code every sample so that name of subject is not shown and that personal information is kept confidential. Based on the information obtained from dietary record, we can determine major essential and trace elements contributing food items in Hong Kong lactating women's diet. We will perform laboratory analyses of on your samples. We will extract the genomic DNA, followed by sequencing and analysis.

7. To be a volunteer in this study we need you to be able to say 'yes' to these points:

Local healthy Chinese lactating mothers (18-40 years old) and whose infants are healthy, vaginal delivery and purely breastfed (aged 2 – 4months). Hong Kong residents having resided in Hong Kong for a continuous period of not less than 18 months; Had normal pre-pregnant BMI (i.e. 19.0-23.5) and weight gain during pregnancy (i.e. 11-16.4kg); Deliver at full term (>37 gestation weeks), give birth to singleton infant within normal birth weight of > 2500g and baby had no known abnormality.

8. **We also need you to be able to say 'no' to these points:**

Concurrent participation in any clinical trial or study; Take any supplements of probiotics during pregnancy and lactation; Take antibiotics for at least 1 month before sample collection; Use contraceptive medication after giving birth; Complicated pregnancy such as preeclampsia and gestational diabetes; Special dietary restrictions for examples gluten-free diets, vegan or any restrictions due to food allergies; Suffer from renal, liver or thyroid dysfunction, cognitive impairment, or any other indication of a major medical or psychological illness, as judged by the investigators as ineligible to participate the study;

Even after you volunteer, you have every right to withdraw from the study before or during the trial without any penalty. All information related to you will remain confidential, and will be identifiable by codes known only to the researcher.
If you have any complaints about the conduct of this research study, please do not hesitate to contact Miss Cherrie Mok, Secretary of the Human Subjects Ethics Sub-Committee of The Hong Kong Polytechnic University in writing (c/o Research Office of the University) stating clearly the responsible person and department of this study.

If you would like more information about this study, please contact **Prof Man-sau Wong on 3400 8665, Dr. Amber Chiou on 3400 8664 or Miss Lilian Yeung on 9357 .**

Thank you for your interest in participating in this study.

WRITTEN CONSENT TO PARTICIPATE IN RESEARCH PROJECT ENTITLED:

The association between maternal diet, early feeding practices and infant gut microbiota: A pilot study is developed specifically to understand the relationship between gut microbiota of purely breastfed infant, breast milk content and maternal diet.

Part 1

I _____ hereby consent my infant and I to participate in the captioned research conducted by Ms. Lilian Yeung , under the supervision of Prof. Man-sau Wong and Dr. Jiachi Chiou.

I understand that the information obtained from this research may be used in future research and published. However, my right to privacy will be retained, i.e., my personal details will not be revealed.

The procedures as set out in the attached information sheet have been fully explained. I understand the benefits and risks involved. My participation in the project is voluntary.

I acknowledge that I have the right to question any part of the procedure and can withdraw at any time without penalty of any kind.

I volunteer to take part in this study.
Name of participant _____
Signature of participant _____
Name of researcher _____
Signature of researcher _____
Date _____

參與者知情同意書

婦女飲食，早期餵養習慣與嬰兒腸道微生物之間的關係：了解全母乳餵養，母乳的營養成分和婦女飲食習慣對嬰兒腸道微生物影響的初步研究

閣下現獲邀參加上述之研究項目。這項研究是由香港理工大學應用生物及化學科技學系黃文秀教授及邱家琪博士負責。以下為此項研究的資料，此資料頁同時設有英文版本，楊藹怡女士樂意以中文或英文為閣下講解此項研究的細則。

1. 研究目的

微生物影響新陳代謝功能和免疫系統。人體帶有上萬億的微生物，尤其腸道中更是存在高密度的微生物。嬰兒的腸道菌群對其腸道健康和未來的發展有直接影響。母乳是嬰兒的第一種營養來源也是能改變嬰兒腸道菌群的因素之一。母乳的營養成分受婦女飲食習慣影響。是次初步研究是為了了解全母乳餵養，母乳的營養成分和婦女飲食習慣對嬰兒腸道微生物的影響。

2. 研究方法：

是次初步研究的參與者為 35 個本地健康婦女（18-40 歲）和她們誕下的健康嬰兒（自然分娩，2- 4 個月全母乳餵養）。我們會收集這些婦女飲食習慣作膳食分析。我們也收集母乳樣本和健康嬰兒的糞便樣本（>1.5 克）測試其中的腸道菌群種類。我們將從這些糞便樣品中提取基因組脫氧核醣核酸、定序該基因組序列。分析和比較不同嬰孩中的腸道菌群種類和母乳營養成分的差異性及相同性。

3. 研究模式：

參與者會參加母乳餵哺研討會。研討會會在香港理工大學舉行而初步研究也會在同一天舉行。研究人員將篩選符合資格的對象，解釋研究細節，並邀請他們參與研究。研究人員亦會邀請每個參與者簽署知情同意書，數據和樣本收集將在研討會當天進行。嬰兒會被要求在研討會開始前換尿布，研究人員會收集糞便樣本並保存在 4℃冰箱中。母親會被安排到獨立房間提供母乳樣本和進行飲食習慣的問卷調查。在研究的前一天，研究人員會再次聯繫提醒參與者於翌日的採訪和樣本採集。所有參與者的樣本將被編上編號以茲識別，絕不會出現參與者名字，樣本編號亦只有相關的研究人員才能解讀，所有參與者的資料必將保密。

4. 研究結果、相關性、意義和價值：

腸道菌群是研究領域的一個新方向，和預防疾病有關。但是，現時並沒有大量香港嬰兒腸道菌群的數據。通過分析嬰兒腸道菌群和母乳的營養成分，我們可以了解它們之間的關聯。母親哺乳期飲食習慣可能會影響母乳中的微生物群或營養成分。因此，如果能發現當中有任何關聯，改變婦女(尤其是過胖的母親)的飲食習慣可能是其中一種降低孩子日後生活疾病的風險的策略。

5. 如您願意參加這項研究，您將要：

A. 簽署知情同意書

B. 填寫飲食問卷調查
C. 為這項研究提供 50 毫升母乳
D. 提供當天嬰兒糞便樣品（>1.5 克）
E. 提供體格資料，如身高體重

請在整個研究中維持日常的飲食習慣

6. 我們將怎樣處理您的嬰兒糞便樣本？

所有參與者的樣本會被編上編號以茲識別，絶不會出現參與者名字，樣本編號亦只有相關的研究人員才能解讀，所有參與者的資料必將保密。根據從飲食記錄獲得的資料，我們可得知香港哺乳期婦女飲食中，哪些是提供人體所需主要及微量元素的主要食物。我們將提取糞便樣品中的基因組脫氧核醣核酸、決定該基因組序列並分析 。

7. 此項研究的對象必須符合以下各項要求：

a. 年齡介乎18-40歲的健康良好的婦女和她們誕下健康嬰兒（2- 4個月全母乳餵養）;
b. 香港居民於香港連續居住不少於 18 個月
c. 產前的體重指標(BMI)屬正常，即介乎 19.0-23.5
d. 懷孕期的體重增長屬正常，即介乎 11-16.4 公斤
e. 足月（> 37孕週）自然分娩，單胎嬰兒及其出生體重正常（> 2500克），健康良好
f. 在研究進行期間不能同時參與其他臨床試驗
g. 在懷孕和哺乳期間並沒有服用任何益生菌的補充劑
h. 參加者或其嬰兒在研究當天的一個月內沒有服用抗生素
i. 生產後沒有服用避孕藥物
j. 沒有任何妊娠併發症，如妊娠毒血症及妊娠糖尿病等
k. 沒有特殊的飲食限制，如無麩質飲食，素食主義者或任何因食物過敏而造成的飲食限制;
l. 沒有腎、肝或甲狀腺功能減退症狀
m. 沒有認知功能障礙
n. 沒有出現經研究人員判斷後認為不適合參加研究的任何身體或精神重大疾病的病徵

當您同意成為參加者後，可於研究開始前或進行期間退出，並不需要負上任何責任。所有有關閣下的資料會保持機密，只有相關的研究人員能夠識別有關資料。

如果您有任何關於此項研究行為的投訴，請以書面形式聯繫香港理工大學研究事務處人類實驗對象操守小組委員會秘書莫小姐，並於信中列明研究負責人名稱及研究部門。

如您想了解這項研究的更多資料，請致電(9357)楊藹怡女士、34008664 邱家琪或 3400 8665 黃文秀教授。

感謝您樂意參與此項研究。

參與者知情同意書

婦女飲食，早期餵養習慣與嬰兒腸道微生物之間的關係：了解全母乳餵養，母乳的營養成分和婦女飲食習慣對嬰兒腸道微生物影響的初步研究

第1部分

本人_____同意與嬰兒共同與這項楊藹怡女士實行並由黃文秀教授及邱家琪博士負責監督的上述研究。

本人知悉此研究所得的資料可能被用作日後的研究及發表，但本人的私隱權利將得以保留，即本人的個人資料不會被公開。

研究人員已向本人清楚解釋列在附頁的資料頁上的研究程序，本人明瞭當中涉及的利益及風險；本人自願參與研究項目。

本人知悉本人有權就程序的任何部分提出疑問，並有權隨時退出而不受任何懲處。
本人自願參與這研究項目。

參與者姓名 _____
參與者簽署 _____
研究人員姓名 _____
研究人員簽署 _____
日期 _____

References

Adair, L. S., & Cole, T. J. (2003). Rapid child growth raises blood pressure in adolescent boys who were thin at birth. *Hypertension*, *41*(3), 451-456. https://doi.org/10.1161/01.HYP.0000054212.23528.B2

Alcock, J., & Lin, H. C. (2015). Fatty acids from diet and microbiota regulate energy metabolism. *F1000Res*, *4*(F1000 Faculty Rev), 738. https://doi.org/10.12688/f1000research.6078.1

Alsharairi, N. A. (2020). The Infant Gut Microbiota and Risk of Asthma: The Effect of Maternal Nutrition during Pregnancy and Lactation. *Microorganisms*, *8*(8). https://doi.org/10.3390/microorganisms8081119

Angeles-Agdeppa, I., Custodio, M. R. S., & Tanda, K. V. (2022). Analysis of food sources and nutrient intakes of selected breastfeeding mothers in Metro Manila, Philippines. *BMC Nutr*, *8*(1), 6. https://doi.org/10.1186/s40795-022-00502-1

Ardissone, A. N., de la Cruz, D. M., Davis-Richardson, A. G., Rechcigl, K. T., Li, N., Drew, J. C., Murgas-Torrazza, R., Sharma, R., Hudak, M. L., Triplett, E. W., & Neu, J. (2014). Meconium microbiome analysis identifies bacteria correlated with premature birth. *PLoS One*, *9*(3), e90784. https://doi.org/10.1371/journal.pone.0090784

Arora, T., & Backhed, F. (2016). The gut microbiota and metabolic disease: current understanding and future perspectives. *J Intern Med*, *280*(4), 339-349. https://doi.org/10.1111/joim.12508

Arrieta, M. C., Arevalo, A., Stiemsma, L., Dimitriu, P., Chico, M. E., Loor, S., Vaca, M., Boutin, R. C. T., Morien, E., Jin, M., Turvey, S. E., Walter, J., Parfrey, L. W., Cooper, P. J., & Finlay, B. (2018). Associations between infant fungal and bacterial dysbiosis and childhood atopic wheeze in a nonindustrialized setting. *J Allergy Clin Immunol*, *142*(2), 424-434 e410. https://doi.org/10.1016/j.jaci.2017.08.041

Arrieta, M. C., Stiemsma, L. T., Amenyogbe, N., Brown, E. M., & Finlay, B. (2014). The intestinal microbiome in early life: health and disease. *Front Immunol*, *5*, 427. https://doi.org/10.3389/fimmu.2014.00427

Arzamasov, A. A., Nakajima, A., Sakanaka, M., Ojima, M. N., Katayama, T., Rodionov, D. A., & Osterman, A. L. (2022). Human Milk Oligosaccharide Utilization in Intestinal Bifidobacteria Is Governed by Global Transcriptional Regulator NagR. *mSystems*, *7*(5), e0034322. https://doi.org/10.1128/msystems.00343-22

Aumeistere, L., Ciprovica, I., Zavadska, D., Andersons, J., Volkovs, V., & Celmalniece, K. (2019). Impact of Maternal Diet on Human Milk Composition Among Lactating Women in Latvia. *Medicina (Kaunas)*, *55*(5). https://doi.org/10.3390/medicina55050173

Azad, M. B., Konya, T., Maughan, H., Guttman, D. S., Field, C. J., Chari, R. S., Sears, M. R., Becker, A. B., Scott, J. A., Kozyrskyj, A. L., & Investigators, C. S. (2013). Gut microbiota of healthy Canadian infants: profiles by mode of delivery and infant diet at 4 months. *CMAJ*, *185*(5), 385-394. https://doi.org/10.1503/cmaj.121189

Babakobi, M. D., Reshef, L., Gihaz, S., Belgorodsky, B., Fishman, A., Bujanover, Y., & Gophna, U. (2020). Effect of Maternal Diet and Milk Lipid Composition on the Infant Gut and Maternal Milk Microbiomes. *Nutrients*, *12*(9). https://doi.org/10.3390/nu12092539

Backhed, F., Roswall, J., Peng, Y., Feng, Q., Jia, H., Kovatcheva-Datchary, P., Li, Y., Xia, Y., Xie, H., Zhong, H., Khan, M. T., Zhang, J., Li, J., Xiao, L., Al-Aama, J., Zhang, D., Lee, Y. S., Kotowska, D., Colding, C., . . . Wang, J. (2015). Dynamics and Stabilization of the Human Gut Microbiome during the First Year of Life. *Cell Host Microbe*, *17*(5), 690-703. https://doi.org/10.1016/j.chom.2015.04.004

Bae, Y. J., & Kratzsch, J. (2018). Vitamin D and calcium in the human breast milk. *Best Pract Res Clin Endocrinol Metab*, *32*(1), 39-45. https://doi.org/10.1016/j.beem.2018.01.007

Ballard, O., & Morrow, A. L. (2013). Human milk composition: nutrients and bioactive factors. *Pediatr Clin North Am*, *60*(1), 49-74. https://doi.org/10.1016/j.pcl.2012.10.002

Barczynska, R., Litwin, M., Slizewska, K., Szalecki, M., Berdowska, A., Bandurska, K., Libudzisz, Z., & Kapusniak, J. (2018). Bacterial Microbiota and Fatty Acids in the Faeces of Overweight and Obese Children. *Pol J Microbiol*, *67*(3), 339-345. https://doi.org/10.21307/pjm-2018-041

Barker, D. J. (1997). Maternal nutrition, fetal nutrition, and disease in later life. *Nutrition*, *13*(9), 807-813. https://doi.org/10.1016/s0899-9007(97)00193-7

Barker, D. J. (2007). The origins of the developmental origins theory. *J Intern Med*, *261*(5), 412-417. https://doi.org/10.1111/j.1365-2796.2007.01809.x

Beam, A., Clinger, E., & Hao, L. (2021). Effect of Diet and Dietary Components on the Composition of the Gut Microbiota. *Nutrients*, *13*(8). https://doi.org/10.3390/nu13082795

Bezirtzoglou, E., Tsiotsias, A., & Welling, G. W. (2011). Microbiota profile in feces of breast- and formula-fed newborns by using fluorescence in situ hybridization (FISH). *Anaerobe*, *17*(6), 478-482. https://doi.org/10.1016/j.anaerobe.2011.03.009

Bharadia, L., Agrawal, N., & Joshi, N. (2020). Development and Functions of the Infant Gut Microflora: Western vs. Indian Infants. *Int J Pediatr*, *2020*, 7586264. https://doi.org/10.1155/2020/7586264

Biddulph, C., Holmes, M., Tran, T. D., Kuballa, A., Davies, P. S. W., Koorts, P., & Maher, J. (2023). Associations between Maternal Nutrition and the Concentrations of Human Milk Oligosaccharides in a Cohort of Healthy Australian Lactating Women. *Nutrients 15*, 2093. https://doi.org/ https://doi.org/10.3390/nu15092093

Bilal, J. A., Rayis, D. A., AlEed, A., Al-Nafeesah, A., & Adam, I. (2022). Maternal Undernutrition and Low Birth Weight in a Tertiary Hospital in Sudan: A Cross-Sectional Study. *Front Pediatr*, *10*, 927518. https://doi.org/10.3389/fped.2022.927518

Bode, L. (2012). Human milk oligosaccharides: every baby needs a sugar mama. *Glycobiology*, *22*(9), 1147-1162. https://doi.org/10.1093/glycob/cws074

Boquien, C. Y. (2018). Human Milk: An Ideal Food for Nutrition of Preterm Newborn. *Front Pediatr*, *6*, 295. https://doi.org/10.3389/fped.2018.00295

Borewicz, K., Gu, F., Saccenti, E., Hechler, C., Beijers, R., de Weerth, C., van Leeuwen, S. S., Schols, H. A., & Smidt, H. (2020). The association between breastmilk oligosaccharides and faecal microbiota in healthy breastfed infants at two, six, and twelve weeks of age. *Sci Rep*, *10*(1), 4270. https://doi.org/10.1038/s41598-020-61024-z

Bravi, F., Wiens, F., Decarli, A., Dal Pont, A., Agostoni, C., & Ferraroni, M. (2016). Impact of maternal nutrition on breast-milk composition: a systematic review. *Am J Clin Nutr*, *104*(3), 646-662. https://doi.org/10.3945/ajcn.115.120881

Butel, M. J., Waligora-Dupriet, A. J., & Wydau-Dematteis, S. (2018). The developing gut microbiota and its consequences for health. *J Dev Orig Health Dis*, *9*(6), 590-597. https://doi.org/10.1017/S2040174418000119

Calder, P. C. (2015). Functional Roles of Fatty Acids and Their Effects on Human Health. *JPEN J Parenter Enteral Nutr*, *39*(1 Suppl), 18S-32S. https://doi.org/10.1177/0148607115595980

Cani, P. D., Amar, J., Iglesias, M. A., Poggi, M., Knauf, C., Bastelica, D., Neyrinck, A. M., Fava, F., Tuohy, K. M., Chabo, C., Waget, A., Delmee, E., Cousin, B., Sulpice, T., Chamontin, B., Ferrieres, J., Tanti, J. F., Gibson, G. R., Casteilla, L., . . . Burcelin, R. (2007). Metabolic endotoxemia initiates obesity and insulin resistance. *Diabetes*, *56*(7), 1761-1772. https://doi.org/10.2337/db06-1491

CDC. (2022). *Maternal Diet.* https://www.cdc.gov/breastfeeding/breastfeeding-special-circumstances/diet-and-micronutrients/maternal-diet.html#:~:text=An%20additional%20330%20to%20400,active%2C%20non%2Dpregnant%20women%20who

Cheema, A. S., Gridneva, Z., Furst, A. J., Roman, A. S., Trevenen, M. L., Turlach, B. A., Lai, C. T., Stinson, L. F., Bode, L., Payne, M. S., & Geddes, D. T. (2022). Human Milk Oligosaccharides and Bacterial Profile Modulate Infant Body Composition during Exclusive Breastfeeding. *Int J Mol Sci*, *23*(5). https://doi.org/10.3390/ijms23052865

Chen, L. W., Tint, M. T., Fortier, M. V., Aris, I. M., Bernard, J. Y., Colega, M., Gluckman, P. D., Saw, S. M., Chong, Y. S., Yap, F., Godfrey, K. M., Kramer, M. S., van Dam, R. M., Chong, M. F., & Lee, Y. S. (2016). Maternal Macronutrient Intake during Pregnancy Is Associated with Neonatal Abdominal Adiposity: The Growing Up in Singapore Towards healthy Outcomes (GUSTO) Study. *J Nutr*, *146*(8), 1571-1579. https://doi.org/10.3945/jn.116.230730

Chen, X. (2015). Human Milk Oligosaccharides (HMOS): Structure, Function, and Enzyme-Catalyzed Synthesis. *Adv Carbohydr Chem Biochem*, *72*, 113-190. https://doi.org/10.1016/bs.accb.2015.08.002

CHP. (2017). *Dietary fibre* https://www.chp.gov.hk/en/static/90018.html

CHP. (2020). *Report of Health Behaviour Survey 2018/19*. Centre of Health Protection. https://www.chp.gov.hk/files/pdf/report_of_health_behaviour_survey_2018_en.pdf

Chu, D. M., Antony, K. M., Ma, J., Prince, A. L., Showalter, L., Moller, M., & Aagaard, K. M. (2016). The early infant gut microbiome varies in association with a maternal high-fat diet. *Genome Med*, *8*(1), 77. https://doi.org/10.1186/s13073-016-0330-z

CNS. (2013). *Chinese Nutrition Society Chinese Dietary Reference Intakes.* People's Medical Publishing House

Collado, M. C., Isolauri, E., Laitinen, K., & Salminen, S. (2010). Effect of mother's weight on infant's microbiota acquisition, composition, and activity during early infancy: a prospective follow-up study initiated in early pregnancy. *Am J Clin Nutr*, *92*(5), 1023-1030. https://doi.org/10.3945/ajcn.2010.29877

Collado, M. C., Rautava, S., Aakko, J., Isolauri, E., & Salminen, S. (2016). Human gut colonisation may be initiated in utero by distinct microbial communities in the placenta and amniotic fluid. *Sci Rep*, *6*, 23129. https://doi.org/10.1038/srep23129

Corona-Cervantes, K., Garcia-Gonzalez, I., Villalobos-Flores, L. E., Hernandez-Quiroz, F., Pina-Escobedo, A., Hoyo-Vadillo, C., Rangel-Calvillo, M. N., & Garcia-Mena, J. (2020). Human milk microbiota associated with early colonization of the neonatal gut in Mexican newborns. *PeerJ*, *8*, e9205. https://doi.org/10.7717/peerj.9205

Cortes-Macias, E., Selma-Royo, M., Garcia-Mantrana, I., Calatayud, M., Gonzalez, S., Martinez-Costa, C., & Collado, M. C. (2021). Maternal Diet Shapes the Breast Milk Microbiota Composition and Diversity: Impact of Mode of Delivery and Antibiotic Exposure. *J Nutr*, *151*(2), 330-340. https://doi.org/10.1093/jn/nxaa310

D'Argenio, V. (2018). The Prenatal Microbiome: A New Player for Human Health. *High Throughput*, *7*(4). https://doi.org/10.3390/ht7040038

Dalby, M. J., & Hall, L. J. (2020). Recent advances in understanding the neonatal microbiome. *F1000Res*, *9*. https://doi.org/10.12688/f1000research.22355.1

DH. (2021). *Report of The Second Hong Kong Population-based Food Consumption Survey*. https://www.cfs.gov.hk/english/programme/programme_firm/files/2nd_FCS_Report_29_Jun_2021.pdf

DH. (2022). *Healthy Eating During Pregnancy and Breastfeeding*. https://www.fhs.gov.hk/english/health_info/woman/20036.html#leaflet-block-wrapper

Ding, Y., Indayati, W., Basnet, T. B., Li, F., Luo, H., Pan, H., & Wang, Z. (2020). Dietary intake in lactating mothers in China 2018: report of a survey. *Nutr J*, *19*(1), 72. https://doi.org/10.1186/s12937-020-00589-x

Ding, Y., Li, F., Hu, P., Ye, M., Xu, F., Jiang, W., Yang, Y., Fu, Y., Zhu, Y., Lu, X., Liu, Y., Xie, Z., & Wang, Z. (2021). Reproducibility and relative validity of a semi-quantitative food frequency questionnaire for the Chinese lactating mothers. *Nutr J*, *20*(1), 20. https://doi.org/10.1186/s12937-021-00678-5

Doherty, A. M., Lodge, C. J., Dharmage, S. C., Dai, X., Bode, L., & Lowe, A. J. (2018). Human Milk Oligosaccharides and Associations With Immune-Mediated Disease and Infection in Childhood: A Systematic Review. *Front Pediatr*, *6*, 91. https://doi.org/10.3389/fped.2018.00091

Drago, L., Panelli, S., Bandi, C., Zuccotti, G., Perini, M., & D'Auria, E. (2019). What Pediatricians Should Know Before Studying Gut Microbiota. *J Clin Med*, *8*(8). https://doi.org/10.3390/jcm8081206

Fallani, M., Young, D., Scott, J., Norin, E., Amarri, S., Adam, R., Aguilera, M., Khanna, S., Gil, A., Edwards, C. A., Dore, J., & Other Members of the, I. T. (2010). Intestinal microbiota of 6-week-old infants across Europe: geographic influence beyond delivery mode, breast-feeding, and antibiotics. *J Pediatr Gastroenterol Nutr*, *51*(1), 77-84. https://doi.org/10.1097/MPG.0b013e3181d1b11e

FAO. (2021). *FAO yearbook. Fishery and Aquaculture Statistics 2019/FAO annuaire. Statistiques des pêches et de l'aquaculture 2019/FAO anuario. Estadísticas de pesca y acuicultura 2019. Rome/Roma.*

Ferreira, A. L., Alves, R., Figueiredo, A., Alves-Santos, N., Freitas-Costa, N., Batalha, M., Yonemitsu, C., Manivong, N., Furst, A., Bode, L., & Kac, G. (2020). Human Milk Oligosaccharide Profile Variation Throughout Postpartum in Healthy Women in a Brazilian Cohort. *Nutrients*, *12*(3). https://doi.org/10.3390/nu12030790

Fields, D. A., & Demerath, E. W. (2012). Relationship of insulin, glucose, leptin, IL-6 and TNF-alpha in human breast milk with infant growth and body composition. *Pediatr Obes*, *7*(4), 304-312. https://doi.org/10.1111/j.2047-6310.2012.00059.x

Fijan, S. (2014). Microorganisms with claimed probiotic properties: an overview of recent literature. *Int J Environ Res Public Health*, *11*(5), 4745-4767. https://doi.org/10.3390/ijerph110504745

Fitzstevens, J. L., Smith, K. C., Hagadorn, J. I., Caimano, M. J., Matson, A. P., & Brownell, E. A. (2017). Systematic Review of the Human Milk Microbiota. *Nutr Clin Pract*, *32*(3), 354-364. https://doi.org/10.1177/0884533616670150

Gagnon, A., Khan, D. R., Sirard, M. A., Girard, C. L., Laforest, J. P., & Richard, F. J. (2015). Effects of intramuscular administration of folic acid and vitamin B12 on granulosa cells gene expression in postpartum dairy cows. *J Dairy Sci*, *98*(11), 7797-7809. https://doi.org/10.3168/jds.2015-9623

Garcia-Mantrana, I., Selma-Royo, M., Gonzalez, S., Parra-Llorca, A., Martinez-Costa, C., & Collado, M. C. (2020). Distinct maternal microbiota clusters are associated with diet during pregnancy: impact on neonatal microbiota and infant growth during the first 18 months of life. *Gut Microbes*, *11*(4), 962-978. https://doi.org/10.1080/19490976.2020.1730294

Gensollen, T., Iyer, S. S., Kasper, D. L., & Blumberg, R. S. (2016). How colonization by microbiota in early life shapes the immune system. *Science*, *352*(6285), 539-544. https://doi.org/10.1126/science.aad9378

Gershuni, V., Li, Y., Elovitz, M., Li, H., Wu, G. D., & Compher, C. W. (2021). Maternal gut microbiota reflecting poor diet quality is associated with spontaneous preterm birth in a prospective cohort study. *Am J Clin Nutr*, *113*(3), 602-611. https://doi.org/10.1093/ajcn/nqaa361

Ghosh, S., DeCoffe, D., Brown, K., Rajendiran, E., Estaki, M., Dai, C., Yip, A., & Gibson, D. L. (2013). Fish oil attenuates omega-6 polyunsaturated fatty acid-induced dysbiosis and infectious colitis but impairs LPS dephosphorylation activity causing sepsis. *PLoS One*, *8*(2), e55468. https://doi.org/10.1371/journal.pone.0055468

Gilley, S. P., Ruebel, M. L., Sims, C., Zhong, Y., Turner, D., Lan, R. S., Pack, L. M., Piccolo, B. D., Chintapalli, S. V., Abraham, A., Bode, L., Andres, A., & Shankar, K. (2022). Associations between maternal obesity and offspring gut microbiome in the first year of life. *Pediatr Obes*, *17*(9), e12921. https://doi.org/10.1111/ijpo.12921

Gronlund, M. M., Lehtonen, O. P., Eerola, E., & Kero, P. (1999). Fecal microflora in healthy infants born by different methods of delivery: permanent changes in intestinal flora after cesarean delivery. *J Pediatr Gastroenterol Nutr*, *28*(1), 19-25. https://doi.org/10.1097/00005176-199901000-00007

Haarman, M., & Knol, J. (2005). Quantitative real-time PCR assays to identify and quantify fecal Bifidobacterium species in infants receiving a prebiotic infant formula. *Appl Environ Microbiol*, *71*(5), 2318-2324. https://doi.org/10.1128/AEM.71.5.2318-2324.2005

Hesla, H. M., Stenius, F., Jaderlund, L., Nelson, R., Engstrand, L., Alm, J., & Dicksved, J. (2014). Impact of lifestyle on the gut microbiota of healthy infants and their mothers-the ALADDIN birth cohort. *FEMS Microbiol Ecol*, *90*(3), 791-801. https://doi.org/10.1111/1574-6941.12434

Higurashi, S., Tsujimori, Y., Nojiri, K., Toba, Y., Nomura, K., & Ueno, H. M. (2023). Dietary Patterns Associated with General Health of Breastfeeding Women 1-2

Months Postpartum: Data from the Japanese Human Milk Study Cohort. *Curr Dev Nutr*, *7*(1), 100004. https://doi.org/10.1016/j.cdnut.2022.100004

Hill, C. J., Lynch, D. B., Murphy, K., Ulaszewska, M., Jeffery, I. B., O'Shea, C. A., Watkins, C., Dempsey, E., Mattivi, F., Tuohy, K., Ross, R. P., Ryan, C. A., PW, O. T., & Stanton, C. (2017). Evolution of gut microbiota composition from birth to 24 weeks in the INFANTMET Cohort. *Microbiome*, *5*(1), 4. https://doi.org/10.1186/s40168-016-0213-y

Ho, N. T., Li, F., Lee-Sarwar, K. A., Tun, H. M., Brown, B. P., Pannaraj, P. S., Bender, J. M., Azad, M. B., Thompson, A. L., Weiss, S. T., Azcarate-Peril, M. A., Litonjua, A. A., Kozyrskyj, A. L., Jaspan, H. B., Aldrovandi, G. M., & Kuhn, L. (2018). Meta-analysis of effects of exclusive breastfeeding on infant gut microbiota across populations. *Nat Commun*, *9*(1), 4169. https://doi.org/10.1038/s41467-018-06473-x

Hong, Q., Ruhaak, L. R., Totten, S. M., Smilowitz, J. T., German, J. B., & Lebrilla, C. B. (2014). Label-free absolute quantitation of oligosaccharides using multiple reaction monitoring. *Anal Chem*, *86*(5), 2640-2647. https://doi.org/10.1021/ac404006z

Imai, C., Takimoto, H., Fudono, A., Tarui, I., Aoyama, T., Yago, S., Okamitsu, M., Sasaki, S., Mizutani, S., Miyasaka, N., & Sato, N. (2021). Application of the Nutrient-Rich Food Index 9.3 and the Dietary Inflammatory Index for Assessing Maternal Dietary Quality in Japan: A Single-Center Birth Cohort Study. *Nutrients*, *13*(8). https://doi.org/10.3390/nu13082854

IOM. (2000). Institute of Medicine (US) Subcommittee on Interpretation and Uses of Dietary Reference Intakes, & Institute of Medicine (US) Standing Committee on the Scientific Evaluation of Dietary Reference Intakes. . In *DRI Dietary Reference Intakes: Applications in Dietary Assessment*. National Academies Press (US). https://doi.org/10.17226/9956

IOM. (2009). *Institute of Medicine (US) and National Research Council (US) Committee to Reexamine IOM Pregnancy Weight Guidelines . Determining optimal weight gain. In: Rasmussen K.M., Yaktine A.L., editors. Weight Gain during Pregnancy: Reexamining the Guidelines. National Academies Press; Washington, DC, USA: 2009.*

Italianer, M. F., Naninck, E. F. G., Roelants, J. A., van der Horst, G. T. J., Reiss, I. K. M., Goudoever, J. B. V., Joosten, K. F. M., Chaves, I., & Vermeulen, M. J. (2020). Circadian Variation in Human Milk Composition, a Systematic Review. *Nutrients*, *12*(8). https://doi.org/10.3390/nu12082328

Jenness, R. (1979). The composition of human milk. *Semin Perinatol*, *3*(3), 225-239. https://www.ncbi.nlm.nih.gov/pubmed/392766

Jeurink, P. V., van Bergenhenegouwen, J., Jimenez, E., Knippels, L. M., Fernandez, L., Garssen, J., Knol, J., Rodriguez, J. M., & Martin, R. (2013). Human milk: a source of more life than we imagine. *Benef Microbes*, *4*(1), 17-30. https://doi.org/10.3920/BM2012.0040

Jiang, T., Liu, B., Li, J., Dong, X., Lin, M., Zhang, M., Zhao, J., Dai, Y., & Chen, L. (2018). Association between sn-2 fatty acid profiles of breast milk and development of the infant intestinal microbiome. *Food Funct*, *9*(2), 1028-1037. https://doi.org/10.1039/c7fo00088j

Jimenez, E., Marin, M. L., Martin, R., Odriozola, J. M., Olivares, M., Xaus, J., Fernandez, L., & Rodriguez, J. M. (2008). Is meconium from healthy newborns actually sterile? *Res Microbiol*, *159*(3), 187-193. https://doi.org/10.1016/j.resmic.2007.12.007

Jost, T., Lacroix, C., Braegger, C., & Chassard, C. (2015). Impact of human milk bacteria and oligosaccharides on neonatal gut microbiota establishment and gut health. *Nutr Rev*, *73*(7), 426-437. https://doi.org/10.1093/nutrit/nuu016

Kankaanpaa, P. E., Salminen, S. J., Isolauri, E., & Lee, Y. K. (2001). The influence of polyunsaturated fatty acids on probiotic growth and adhesion. *FEMS Microbiol Lett*, *194*(2), 149-153. https://doi.org/10.1111/j.1574-6968.2001.tb09460.x

Kashtanova, D. A., Popenko, A. S., Tkacheva, O. N., Tyakht, A. B., Alexeev, D. G., & Boytsov, S. A. (2016). Association between the gut microbiota and diet: Fetal life, early childhood, and further life. *Nutrition*, *32*(6), 620-627. https://doi.org/10.1016/j.nut.2015.12.037

Kent, J. C., Arthur, P. G., Mitoulas, L. R., & Hartmann, P. E. (2009). Why calcium in breastmilk is independent of maternal dietary calcium and vitamin D. *Breastfeed Rev*, *17*(2), 5-11. https://www.ncbi.nlm.nih.gov/pubmed/19685853

Kobata, A. (2010). Structures and application of oligosaccharides in human milk. *Proc Jpn Acad Ser B Phys Biol Sci*, *86*(7), 731-747. https://doi.org/10.2183/pjab.86.731

Koletzko, B., Brands, B., Chourdakis, M., Cramer, S., Grote, V., Hellmuth, C., Kirchberg, F., Prell, C., Rzehak, P., Uhl, O., & Weber, M. (2014). The Power of Programming and the EarlyNutrition project: opportunities for health promotion by nutrition during the first thousand days of life and beyond. *Ann Nutr Metab*, *64*(3-4), 187-196. https://doi.org/10.1159/000365017

Koletzko, B., Godfrey, K. M., Poston, L., Szajewska, H., van Goudoever, J. B., de Waard, M., Brands, B., Grivell, R. M., Deussen, A. R., Dodd, J. M., Patro-Golab, B., Zalewski, B. M., & EarlyNutrition Project Systematic Review, G. (2019). Nutrition During Pregnancy, Lactation and Early Childhood and its Implications for Maternal and Long-Term Child Health: The Early Nutrition Project Recommendations. *Ann Nutr Metab*, *74*(2), 93-106. https://doi.org/10.1159/000496471

Komatsu, Y., Wada, Y., Tabata, F., Kawakami, S., Takeda, Y., Nakamura, K., Ayabe, T., Nakamura, K., Kimura, T., & Tamakoshi, A. (2023). Associations between Maternal Diet, Human Milk Macronutrients, and Breast-Fed Infant Growth during the First Month of Life in the SMILE Iwamizawa in Japan. *Nutrients*, *15*(3). https://doi.org/10.3390/nu15030654

Kovatcheva-Datchary, P., Nilsson, A., Akrami, R., Lee, Y. S., De Vadder, F., Arora, T., Hallen, A., Martens, E., Bjorck, I., & Backhed, F. (2015). Dietary Fiber-Induced Improvement in Glucose Metabolism Is Associated with Increased Abundance of Prevotella. *Cell Metab*, *22*(6), 971-982. https://doi.org/10.1016/j.cmet.2015.10.001

Kunz, C., Meyer, C., Collado, M. C., Geiger, L., Garcia-Mantrana, I., Bertua-Rios, B., Martinez-Costa, C., Borsch, C., & Rudloff, S. (2017). Influence of Gestational Age, Secretor, and Lewis Blood Group Status on the Oligosaccharide Content of Human Milk. *J Pediatr Gastroenterol Nutr*, *64*(5), 789-798. https://doi.org/10.1097/MPG.0000000000001402

Kunz, C., Rudloff, S., Baier, W., Klein, N., & Strobel, S. (2000). Oligosaccharides in human milk: structural, functional, and metabolic aspects. *Annu Rev Nutr*, *20*, 699-722. https://doi.org/10.1146/annurev.nutr.20.1.699

Larsen, N., Vogensen, F. K., van den Berg, F. W., Nielsen, D. S., Andreasen, A. S., Pedersen, B. K., Al-Soud, W. A., Sorensen, S. J., Hansen, L. H., & Jakobsen, M. (2010). Gut microbiota in human adults with type 2 diabetes differs from

non-diabetic adults. *PLoS One*, *5*(2), e9085. https://doi.org/10.1371/journal.pone.0009085

Lee, H. S. (2019). The interaction between gut microbiome and nutrients on development of human disease through epigenetic mechanisms. *Genomics Inform*, *17*(3), e24. https://doi.org/10.5808/GI.2019.17.3.e24

Lemas, D. J., Young, B. E., Baker, P. R., 2nd, Tomczik, A. C., Soderborg, T. K., Hernandez, T. L., de la Houssaye, B. A., Robertson, C. E., Rudolph, M. C., Ir, D., Patinkin, Z. W., Krebs, N. F., Santorico, S. A., Weir, T., Barbour, L. A., Frank, D. N., & Friedman, J. E. (2016). Alterations in human milk leptin and insulin are associated with early changes in the infant intestinal microbiome. *Am J Clin Nutr*, *103*(5), 1291-1300. https://doi.org/10.3945/ajcn.115.126375

LeMay-Nedjelski, L., Asbury, M. R., Butcher, J., Ley, S. H., Hanley, A. J., Kiss, A., Unger, S., Copeland, J. K., Wang, P. W., Stintzi, A., & O'Connor, D. L. (2021). Maternal Diet and Infant Feeding Practices Are Associated with Variation in the Human Milk Microbiota at 3 Months Postpartum in a Cohort of Women with High Rates of Gestational Glucose Intolerance. *J Nutr*, *151*(2), 320-329. https://doi.org/10.1093/jn/nxaa248

Li, X., Mao, Y., Liu, S., Wang, J., Li, X., Zhao, Y., Hill, D. R., & Wang, S. (2022). Vitamins, Vegetables and Metal Elements Are Positively Associated with Breast Milk Oligosaccharide Composition among Mothers in Tianjin, China. *Nutrients*, *14*(19). https://doi.org/10.3390/nu14194131

Liu, F. L., Zhang, Y. M., Pares, G. V., Reidy, K. C., Zhao, W. Z., Zhao, A., Chen, C., Ning, C. Y., Zheng, Y. D., & Wang, P. Y. (2015). Nutrient Intakes of Pregnant Women and their Associated Factors in Eight Cities of China: A Cross-sectional Study. *Chin Med J (Engl)*, *128*(13), 1778-1786. https://doi.org/10.4103/0366-6999.159354

Lu, Z., Chan, Y. T., Lo, K. K., Zhao, D., Wong, V. W., Ng, Y. F., Ho, W. W., Li, L. S., Lee, H. W., Wong, M. S., & Li, S. Y. (2022). Carotenoids and Vitamin A in Breastmilk of Hong Kong Lactating Mothers and Their Relationships with Maternal Diet. *Nutrients*, *14*(10). https://doi.org/10.3390/nu14102031

Luna, E., Parkar, S. G., Kirmiz, N., Hartel, S., Hearn, E., Hossine, M., Kurdian, A., Mendoza, C., Orr, K., Padilla, L., Ramirez, K., Salcedo, P., Serrano, E., Choudhury, B., Paulchakrabarti, M., Parker, C. T., Huynh, S., Cooper, K., & Flores, G. E. (2022). Utilization Efficiency of Human Milk Oligosaccharides by Human-Associated Akkermansia Is Strain Dependent. *Appl Environ Microbiol*, *88*(1), e0148721. https://doi.org/10.1128/AEM.01487-21

Lundgren, S. N., Madan, J. C., Emond, J. A., Morrison, H. G., Christensen, B. C., Karagas, M. R., & Hoen, A. G. (2018). Maternal diet during pregnancy is related with the infant stool microbiome in a delivery mode-dependent manner. *Microbiome*, *6*(1), 109. https://doi.org/10.1186/s40168-018-0490-8

Lyons, K. E., Ryan, C. A., Dempsey, E. M., Ross, R. P., & Stanton, C. (2020). Breast Milk, a Source of Beneficial Microbes and Associated Benefits for Infant Health. *Nutrients*, *12*(4). https://doi.org/10.3390/nu12041039

Macpherson, A. J., de Aguero, M. G., & Ganal-Vonarburg, S. C. (2017). How nutrition and the maternal microbiota shape the neonatal immune system. *Nat Rev Immunol*, *17*(8), 508-517. https://doi.org/10.1038/nri.2017.58

Marcobal, A., Barboza, M., Sonnenburg, E. D., Pudlo, N., Martens, E. C., Desai, P., Lebrilla, C. B., Weimer, B. C., Mills, D. A., German, J. B., & Sonnenburg, J. L. (2011). Bacteroides in the infant gut consume milk oligosaccharides via mucus-

utilization pathways. *Cell Host Microbe*, *10*(5), 507-514. https://doi.org/10.1016/j.chom.2011.10.007

Marshall, N. E., Abrams, B., Barbour, L. A., Catalano, P., Christian, P., Friedman, J. E., Hay, W. W., Jr., Hernandez, T. L., Krebs, N. F., Oken, E., Purnell, J. Q., Roberts, J. M., Soltani, H., Wallace, J., & Thornburg, K. L. (2022). The importance of nutrition in pregnancy and lactation: lifelong consequences. *Am J Obstet Gynecol*, *226*(5), 607-632. https://doi.org/10.1016/j.ajog.2021.12.035

Martin, R., Langa, S., Reviriego, C., Jiminez, E., Marin, M. L., Xaus, J., Fernandez, L., & Rodriguez, J. M. (2003). Human milk is a source of lactic acid bacteria for the infant gut. *J Pediatr*, *143*(6), 754-758. https://doi.org/10.1016/j.jpeds.2003.09.028

Martin, R., Makino, H., Cetinyurek Yavuz, A., Ben-Amor, K., Roelofs, M., Ishikawa, E., Kubota, H., Swinkels, S., Sakai, T., Oishi, K., Kushiro, A., & Knol, J. (2016). Early-Life Events, Including Mode of Delivery and Type of Feeding, Siblings and Gender, Shape the Developing Gut Microbiota. *PLoS One*, *11*(6), e0158498. https://doi.org/10.1371/journal.pone.0158498

Milani, C., Duranti, S., Bottacini, F., Casey, E., Turroni, F., Mahony, J., Belzer, C., Delgado Palacio, S., Arboleya Montes, S., Mancabelli, L., Lugli, G. A., Rodriguez, J. M., Bode, L., de Vos, W., Gueimonde, M., Margolles, A., van Sinderen, D., & Ventura, M. (2017). The First Microbial Colonizers of the Human Gut: Composition, Activities, and Health Implications of the Infant Gut Microbiota. *Microbiol Mol Biol Rev*, *81*(4). https://doi.org/10.1128/MMBR.00036-17

Minihane, A. M., Vinoy, S., Russell, W. R., Baka, A., Roche, H. M., Tuohy, K. M., Teeling, J. L., Blaak, E. E., Fenech, M., Vauzour, D., McArdle, H. J., Kremer, B. H., Sterkman, L., Vafeiadou, K., Benedetti, M. M., Williams, C. M., & Calder, P. C. (2015). Low-grade inflammation, diet composition and health: current research evidence and its translation. *Br J Nutr*, *114*(7), 999-1012. https://doi.org/10.1017/S0007114515002093

Mitsou, E. K., Kirtzalidou, E., Oikonomou, I., Liosis, G., & Kyriacou, A. (2008). Fecal microflora of Greek healthy neonates. *Anaerobe*, *14*(2), 94-101. https://doi.org/10.1016/j.anaerobe.2007.11.002

Moore, R. E., & Townsend, S. D. (2019). Temporal development of the infant gut microbiome. *Open Biol*, *9*(9), 190128. https://doi.org/10.1098/rsob.190128

Moossavi, S., Atakora, F., Miliku, K., Sepehri, S., Robertson, B., Duan, Q. L., Becker, A. B., Mandhane, P. J., Turvey, S. E., Moraes, T. J., Lefebvre, D. L., Sears, M. R., Subbarao, P., Field, C. J., Bode, L., Khafipour, E., & Azad, M. B. (2019). Integrated Analysis of Human Milk Microbiota With Oligosaccharides and Fatty Acids in the CHILD Cohort. *Front Nutr*, *6*, 58. https://doi.org/10.3389/fnut.2019.00058

Moossavi, S., Miliku, K., Sepehri, S., Khafipour, E., & Azad, M. B. (2018). The Prebiotic and Probiotic Properties of Human Milk: Implications for Infant Immune Development and Pediatric Asthma. *Front Pediatr*, *6*, 197. https://doi.org/10.3389/fped.2018.00197

Mueller, N. T., Bakacs, E., Combellick, J., Grigoryan, Z., & Dominguez-Bello, M. G. (2015). The infant microbiome development: mom matters. *Trends Mol Med*, *21*(2), 109-117. https://doi.org/10.1016/j.molmed.2014.12.002

Murphy, K., Curley, D., O'Callaghan, T. F., O'Shea, C. A., Dempsey, E. M., O'Toole, P. W., Ross, R. P., Ryan, C. A., & Stanton, C. (2017). The Composition of

Human Milk and Infant Faecal Microbiota Over the First Three Months of Life: A Pilot Study. *Sci Rep*, *7*, 40597. https://doi.org/10.1038/srep40597

Natividad, J. M., Marsaux, B., Rodenas, C. L. G., Rytz, A., Vandevijver, G., Marzorati, M., Van den Abbeele, P., Calatayud, M., & Rochat, F. (2022). Human Milk Oligosaccharides and Lactose Differentially Affect Infant Gut Microbiota and Intestinal Barrier In Vitro. *Nutrients*, *14*(12). https://doi.org/10.3390/nu14122546

Neu, J. (2016). Intestinal Microbiota Studies in Preterm Infants. *J Pediatr Gastroenterol Nutr*, *62*(2), 193-194. https://doi.org/10.1097/MPG.0000000000001068

Newburg, D. S., Ruiz-Palacios, G. M., Altaye, M., Chaturvedi, P., Guerrero, M. L., Meinzen-Derr, J. K., & Morrow, A. L. (2004). Human milk alphal,2-linked fucosylated oligosaccharides decrease risk of diarrhea due to stable toxin of E. coli in breastfed infants. *Adv Exp Med Biol*, *554*, 457-461. https://doi.org/10.1007/978-1-4757-4242-8_64

Niu, J., Xu, L., Qian, Y., Sun, Z., Yu, D., Huang, J., Zhou, X., Wang, Y., Zhang, T., Ren, R., Li, Z., Yu, J., & Gao, X. (2020). Evolution of the Gut Microbiome in Early Childhood: A Cross-Sectional Study of Chinese Children. *Front Microbiol*, *11*, 439. https://doi.org/10.3389/fmicb.2020.00439

Nyangahu, D. D., & Jaspan, H. B. (2019). Influence of maternal microbiota during pregnancy on infant immunity. *Clin Exp Immunol*, *198*(1), 47-56. https://doi.org/10.1111/cei.13331

Nykjaer, C., Higgs, C., Greenwood, D. C., Simpson, N. A. B., Cade, J. E., & Alwan, N. A. (2019). Maternal Fatty Fish Intake Prior to and during Pregnancy and Risks of Adverse Birth Outcomes: Findings from a British Cohort. *Nutrients*, *11*(3). https://doi.org/10.3390/nu11030643

OASH. (2022). *Nutrition During Pregnancy to Support a Healthy Mom and Baby*. https://health.gov/news/202202/nutrition-during-pregnancy-support-healthy-mom-and-baby#:~:text=weight%20gain%20guidelines-,during%20pregnancy.,that%20is%20overweight%20or%20obese.

Odiase, E., Frank, D., Young, B., Robertson, C., Kofonow, J., Davis, K., Berman, L., Krebs, N., & Tang, M. (2022). Gut Microbiota Is Significantly Impacted by Feeding Mode and Associated With Growth Status in Exclusively Breastfed and Formula-Fed US Infants. . *Current Developments in Nutrition*, *6(Suppl 1)*, 1022.

Olin, A., Henckel, E., Chen, Y., Lakshmikanth, T., Pou, C., Mikes, J., Gustafsson, A., Bernhardsson, A. K., Zhang, C., Bohlin, K., & Brodin, P. (2018). Stereotypic Immune System Development in Newborn Children. *Cell*, *174*(5), 1277-1292 e1214. https://doi.org/10.1016/j.cell.2018.06.045

Oria, M. P., & Kumanyika, S. (2017). Guiding Principles for Developing Dietary Reference Intakes Based on Chronic Disease. In. https://doi.org/10.17226/24828

Padilha, M., Danneskiold-Samsoe, N. B., Brejnrod, A., Hoffmann, C., Cabral, V. P., Iaucci, J. M., Sales, C. H., Fisberg, R. M., Cortez, R. V., Brix, S., Taddei, C. R., Kristiansen, K., & Saad, S. M. I. (2019). The Human Milk Microbiota is Modulated by Maternal Diet. *Microorganisms*, *7*(11). https://doi.org/10.3390/microorganisms7110502

Padmanabhan, V., Cardoso, R. C., & Puttabyatappa, M. (2016). Developmental Programming, a Pathway to Disease. *Endocrinology*, *157*(4), 1328-1340. https://doi.org/10.1210/en.2016-1003

Penders, J., Thijs, C., Vink, C., Stelma, F. F., Snijders, B., Kummeling, I., van den Brandt, P. A., & Stobberingh, E. E. (2006). Factors influencing the composition of the intestinal microbiota in early infancy. *Pediatrics*, *118*(2), 511-521. https://doi.org/10.1542/peds.2005-2824

Perez-Munoz, M. E., Arrieta, M. C., Ramer-Tait, A. E., & Walter, J. (2017). A critical assessment of the "sterile womb" and "in utero colonization" hypotheses: implications for research on the pioneer infant microbiome. *Microbiome*, *5*(1), 48. https://doi.org/10.1186/s40168-017-0268-4

Perng, W., Oken, E., & Dabelea, D. (2019). Developmental overnutrition and obesity and type 2 diabetes in offspring. *Diabetologia*, *62*(10), 1779-1788. https://doi.org/10.1007/s00125-019-4914-1

Plaza-Diaz, J., Fontana, L., & Gil, A. (2018). Human Milk Oligosaccharides and Immune System Development. *Nutrients*, *10*(8). https://doi.org/10.3390/nu10081038

Plows, J. F., Berger, P. K., Jones, R. B., Alderete, T. L., Yonemitsu, C., Najera, J. A., Khwajazada, S., Bode, L., & Goran, M. I. (2021). Longitudinal Changes in Human Milk Oligosaccharides (HMOs) Over the Course of 24 Months of Lactation. *J Nutr*, *151*(4), 876-882. https://doi.org/10.1093/jn/nxaa427

Poeker, S. A., Geirnaert, A., Berchtold, L., Greppi, A., Krych, L., Steinert, R. E., de Wouters, T., & Lacroix, C. (2018). Understanding the prebiotic potential of different dietary fibers using an in vitro continuous adult fermentation model (PolyFermS). *Sci Rep*, *8*(1), 4318. https://doi.org/10.1038/s41598-018-22438-y

Polansky, O., Sekelova, Z., Faldynova, M., Sebkova, A., Sisak, F., & Rychlik, I. (2015). Important Metabolic Pathways and Biological Processes Expressed by Chicken Cecal Microbiota. *Appl Environ Microbiol*, *82*(5), 1569-1576. https://doi.org/10.1128/AEM.03473-15

Popkin, B. M., Richards, M. K., & Montiero, C. A. (1996). Stunting is associated with overweight in children of four nations that are undergoing the nutrition transition. *J Nutr*, *126*(12), 3009-3016. https://doi.org/10.1093/jn/126.12.3009

Preynat, A., Lapierre, H., Thivierge, M. C., Palin, M. F., Matte, J. J., Desrochers, A., & Girard, C. L. (2009). Effects of supplements of folic acid, vitamin B12, and rumen-protected methionine on whole body metabolism of methionine and glucose in lactating dairy cows. *J Dairy Sci*, *92*(2), 677-689. https://doi.org/10.3168/jds.2008-1525

Qiao, Y., Feng, J., Yang, J., & Gu, G. (2013). The relationship between dietary vitamin A intake and the levels of sialic acid in the breast milk of lactating women. *J Nutr Sci Vitaminol (Tokyo)*, *59*(4), 347-351. https://doi.org/10.3177/jnsv.59.347

Qin, J., Li, Y., Cai, Z., Li, S., Zhu, J., Zhang, F., Liang, S., Zhang, W., Guan, Y., Shen, D., Peng, Y., Zhang, D., Jie, Z., Wu, W., Qin, Y., Xue, W., Li, J., Han, L., Lu, D., . . . Wang, J. (2012). A metagenome-wide association study of gut microbiota in type 2 diabetes. *Nature*, *490*(7418), 55-60. https://doi.org/10.1038/nature11450

Quin, C., Vicaretti, S. D., Mohtarudin, N. A., Garner, A. M., Vollman, D. M., Gibson, D. L., & Zandberg, W. F. (2020). Influence of sulfonated and diet-derived human milk oligosaccharides on the infant microbiome and immune markers. *J Biol Chem*, *295*(12), 4035-4048. https://doi.org/10.1074/jbc.RA119.011351

Reader, D., & Franz, M. J. (2004). Lactation, diabetes, and nutrition recommendations. *Curr Diab Rep*, *4*(5), 370-376. https://doi.org/10.1007/s11892-004-0040-6

Rinninella, E., Raoul, P., Cintoni, M., Franceschi, F., Miggiano, G. A. D., Gasbarrini, A., & Mele, M. C. (2019). What is the Healthy Gut Microbiota Composition?

A Changing Ecosystem across Age, Environment, Diet, and Diseases. *Microorganisms*, *7*(1). https://doi.org/10.3390/microorganisms7010014

Rizzatti, G., Lopetuso, L. R., Gibiino, G., Binda, C., & Gasbarrini, A. (2017). Proteobacteria: A Common Factor in Human Diseases. *Biomed Res Int*, *2017*, 9351507. https://doi.org/10.1155/2017/9351507

Ruiz-Ojeda, F. J., Plaza-Diaz, J., Morales, J., Alvarez-Calatayud, G., Climent, E., Silva, A., Martinez-Blanch, J. F., Enrique, M., Tortajada, M., Ramon, D., Alvarez, B., Chenoll, E., & Gil, A. (2023). Effects of a Novel Infant Formula on the Fecal Microbiota in the First Six Months of Life: The INNOVA 2020 Study. *Int J Mol Sci*, *24*(3). https://doi.org/10.3390/ijms24033034

Ruiz, L., Delgado, S., Ruas-Madiedo, P., Sanchez, B., & Margolles, A. (2017). Bifidobacteria and Their Molecular Communication with the Immune System. *Front Microbiol*, *8*, 2345. https://doi.org/10.3389/fmicb.2017.02345

Rutayisire, E., Huang, K., Liu, Y., & Tao, F. (2016). The mode of delivery affects the diversity and colonization pattern of the gut microbiota during the first year of infants' life: a systematic review. *BMC Gastroenterol*, *16*(1), 86. https://doi.org/10.1186/s12876-016-0498-0

Sakwinska, O., Moine, D., Delley, M., Combremont, S., Rezzonico, E., Descombes, P., Vinyes-Pares, G., Zhang, Y., Wang, P., & Thakkar, S. K. (2016). Microbiota in Breast Milk of Chinese Lactating Mothers. *PLoS One*, *11*(8), e0160856. https://doi.org/10.1371/journal.pone.0160856

Sanli, E., & Kabaran, S. (2019). Maternal Obesity, Maternal Overnutrition and Fetal Programming: Effects of Epigenetic Mechanisms on the Development of Metabolic Disorders. *Curr Genomics*, *20*(6), 419-427. https://doi.org/10.2174/1389202920666191030092225

Schober, P., Boer, C., & Schwarte, L. A. (2018). Correlation Coefficients: Appropriate Use and Interpretation. *Anesth Analg*, *126*(5), 1763-1768. https://doi.org/10.1213/ANE.0000000000002864

Seferovic, M. D., Mohammad, M., Pace, R. M., Engevik, M., Versalovic, J., Bode, L., Haymond, M., & Aagaard, K. M. (2020). Maternal diet alters human milk oligosaccharide composition with implications for the milk metagenome. *Sci Rep*, *10*(1), 22092. https://doi.org/10.1038/s41598-020-79022-6

Sekerel, B. E., Bingol, G., Cullu Cokugras, F., Cokugras, H., Kansu, A., Ozen, H., & Tamay, Z. (2021). An Expert Panel Statement on the Beneficial Effects of Human Milk Oligosaccharides (HMOs) in Early Life and Potential Utility of HMO-Supplemented Infant Formula in Cow's Milk Protein Allergy. *J Asthma Allergy*, *14*, 1147-1164. https://doi.org/10.2147/JAA.S323734

Selma-Royo, M., Gonzalez, S., Gueimonde, M., Chang, M., Furst, A., Martinez-Costa, C., Bode, L., & Collado, M. C. (2022). Maternal Diet Is Associated with Human Milk Oligosaccharide Profile. *Mol Nutr Food Res*, *66*(15), e2200058. https://doi.org/10.1002/mnfr.202200058

Serino, M., Nicolas, S., Trabelsi, M. S., Burcelin, R., & Blasco-Baque, V. (2017). Young microbes for adult obesity. *Pediatr Obes*, *12*(4), e28-e32. https://doi.org/10.1111/ijpo.12146

Simione, M., Harshman, S. G., Castro, I., Linnemann, R., Roche, B., Ajami, N. J., Petrosino, J. F., Raspini, B., Portale, S., Camargo, C. A., Jr., Taveras, E. M., Hasegawa, K., & Fiechtner, L. (2020). Maternal Fish Consumption in Pregnancy Is Associated with a Bifidobacterium-Dominant Microbiome Profile in Infants. *Curr Dev Nutr*, *4*(1), nzz133. https://doi.org/10.1093/cdn/nzz133

Simon, P. M., Goode, P. L., Mobasseri, A., & Zopf, D. (1997). Inhibition of Helicobacter pylori binding to gastrointestinal epithelial cells by sialic acid-containing oligosaccharides. *Infect Immun*, *65*(2), 750-757. https://doi.org/10.1128/iai.65.2.750-757.1997

Sindi, A. S., Geddes, D. T., Wlodek, M. E., Muhlhausler, B. S., Payne, M. S., & Stinson, L. F. (2021). Can we modulate the breastfed infant gut microbiota through maternal diet? *FEMS Microbiol Rev*, *45*(5). https://doi.org/10.1093/femsre/fuab011

Sindi, A. S., Stinson, L. F., Lean, S. S., Chooi, Y. H., Leghi, G. E., Netting, M. J., Wlodek, M. E., Muhlhausler, B. S., Geddes, D. T., & Payne, M. S. (2022). Effect of a reduced fat and sugar maternal dietary intervention during lactation on the infant gut microbiome. *Front Microbiol*, *13*, 900702. https://doi.org/10.3389/fmicb.2022.900702

Solis, G., de Los Reyes-Gavilan, C. G., Fernandez, N., Margolles, A., & Gueimonde, M. (2010). Establishment and development of lactic acid bacteria and bifidobacteria microbiota in breast-milk and the infant gut. *Anaerobe*, *16*(3), 307-310. https://doi.org/10.1016/j.anaerobe.2010.02.004

Soyyilmaz, B., Miks, M. H., Rohrig, C. H., Matwiejuk, M., Meszaros-Matwiejuk, A., & Vigsnaes, L. K. (2021). The Mean of Milk: A Review of Human Milk Oligosaccharide Concentrations throughout Lactation. *Nutrients*, *13*(8). https://doi.org/10.3390/nu13082737

Sprenger, N., Lee, L. Y., De Castro, C. A., Steenhout, P., & Thakkar, S. K. (2017). Longitudinal change of selected human milk oligosaccharides and association to infants' growth, an observatory, single center, longitudinal cohort study. *PLoS One*, *12*(2), e0171814. https://doi.org/10.1371/journal.pone.0171814

Stark, P. L., & Lee, A. (1982). The microbial ecology of the large bowel of breast-fed and formula-fed infants during the first year of life. *J Med Microbiol*, *15*(2), 189-203. https://doi.org/10.1099/00222615-15-2-189

Stein, A. D., Obrutu, O. E., Behere, R. V., & Yajnik, C. S. (2019). Developmental undernutrition, offspring obesity and type 2 diabetes. *Diabetologia*, *62*(10), 1773-1778. https://doi.org/10.1007/s00125-019-4930-1

Stewart, C. J., Ajami, N. J., O'Brien, J. L., Hutchinson, D. S., Smith, D. P., Wong, M. C., Ross, M. C., Lloyd, R. E., Doddapaneni, H., Metcalf, G. A., Muzny, D., Gibbs, R. A., Vatanen, T., Huttenhower, C., Xavier, R. J., Rewers, M., Hagopian, W., Toppari, J., Ziegler, A. G., . . . Petrosino, J. F. (2018). Temporal development of the gut microbiome in early childhood from the TEDDY study. *Nature*, *562*(7728), 583-588. https://doi.org/10.1038/s41586-018-0617-x

Tam, W. H., Chan, R. S., Chan, M. H., Yuen, L. Y., Li, L., Sea, M. M., & Woo, J. (2017). Moderate iodine deficiency among pregnant women in Hong Kong: revisit the problem after two decades. *Hong Kong Med J*, *23*(6), 586-593. https://doi.org/10.12809/hkmj176841

Tamburini, S., Shen, N., Wu, H. C., & Clemente, J. C. (2016). The microbiome in early life: implications for health outcomes. *Nat Med*, *22*(7), 713-722. https://doi.org/10.1038/nm.4142

Tanaka, M., & Nakayama, J. (2017). Development of the gut microbiota in infancy and its impact on health in later life. *Allergol Int*, *66*(4), 515-522. https://doi.org/10.1016/j.alit.2017.07.010

Toemen, L., Gishti, O., van Osch-Gevers, L., Steegers, E. A., Helbing, W. A., Felix, J. F., Reiss, I. K., Duijts, L., Gaillard, R., & Jaddoe, V. W. (2016). Maternal obesity, gestational weight gain and childhood cardiac outcomes: role of

childhood body mass index. *Int J Obes (Lond)*, *40*(7), 1070-1078. https://doi.org/10.1038/ijo.2016.86

Tomova, A., Bukovsky, I., Rembert, E., Yonas, W., Alwarith, J., Barnard, N. D., & Kahleova, H. (2019). The Effects of Vegetarian and Vegan Diets on Gut Microbiota. *Front Nutr*, *6*, 47. https://doi.org/10.3389/fnut.2019.00047

Totten, S. M., Zivkovic, A. M., Wu, S., Ngyuen, U., Freeman, S. L., Ruhaak, L. R., Darboe, M. K., German, J. B., Prentice, A. M., & Lebrilla, C. B. (2012). Comprehensive profiles of human milk oligosaccharides yield highly sensitive and specific markers for determining secretor status in lactating mothers. *J Proteome Res*, *11*(12), 6124-6133. https://doi.org/10.1021/pr300769g

Trumbo, P., Schlicker, S., Yates, A. A., Poos, M., Food, & Nutrition Board of the Institute of Medicine, T. N. A. (2002). Dietary reference intakes for energy, carbohydrate, fiber, fat, fatty acids, cholesterol, protein and amino acids. *J Am Diet Assoc*, *102*(11), 1621-1630. https://doi.org/10.1016/s0002-8223(02)90346-9

Trumbo, P., Yates, A. A., Schlicker, S., & Poos, M. (2001). Dietary reference intakes: vitamin A, vitamin K, arsenic, boron, chromium, copper, iodine, iron, manganese, molybdenum, nickel, silicon, vanadium, and zinc. *J Am Diet Assoc*, *101*(3), 294-301. https://doi.org/10.1016/S0002-8223(01)00078-5

Tsoi, K. Y., Chan, R. S. M., Tam, C. H. T., Li, L. S., Tam, W. H., & Ma, R. C. W. (2022). Dietary patterns of Chinese pregnant women in Hong Kong. *Asia Pac J Clin Nutr*, *31*(3), 378-393. https://doi.org/10.6133/apjcn.202209_31(3).0006

USDA. (2016a). *Tips for Breastfeeding Moms*. https://wicworks.fns.usda.gov/sites/default/files/media/document/Tips_for_Breastfeeding_Moms_English.pdf

USDA. (2016b). *Tips for Pregnant Moms*. https://wicworks.fns.usda.gov/sites/default/files/media/document/Tips_for_Pregnant_Women_English.pdf

Vandenplas, Y., Berger, B., Carnielli, V. P., Ksiazyk, J., Lagstrom, H., Sanchez Luna, M., Migacheva, N., Mosselmans, J. M., Picaud, J. C., Possner, M., Singhal, A., & Wabitsch, M. (2018). Human Milk Oligosaccharides: 2'-Fucosyllactose (2'-FL) and Lacto-N-Neotetraose (LNnT) in Infant Formula. *Nutrients*, *10*(9). https://doi.org/10.3390/nu10091161

Verdam, F. J., Fuentes, S., de Jonge, C., Zoetendal, E. G., Erbil, R., Greve, J. W., Buurman, W. A., de Vos, W. M., & Rensen, S. S. (2013). Human intestinal microbiota composition is associated with local and systemic inflammation in obesity. *Obesity (Silver Spring)*, *21*(12), E607-615. https://doi.org/10.1002/oby.20466

Walker, R. W., Clemente, J. C., Peter, I., & Loos, R. J. F. (2017). The prenatal gut microbiome: are we colonized with bacteria in utero? *Pediatr Obes*, *12 Suppl 1*, 3-17. https://doi.org/10.1111/ijpo.12217

Walsh, C., Lane, J. A., van Sinderen, D., & Hickey, R. M. (2020). Human milk oligosaccharides: Shaping the infant gut microbiota and supporting health. *J Funct Foods*, *72*, 104074. https://doi.org/10.1016/j.jff.2020.104074

Wan, Z., Zheng, J., Zhu, Z., Sang, L., Zhu, J., Luo, S., Zhao, Y., Wang, R., Zhang, Y., Hao, K., Chen, L., Du, J., Kan, J., & He, H. (2022). Intermediate role of gut microbiota in vitamin B nutrition and its influences on human health. *Front Nutr*, *9*, 1031502. https://doi.org/10.3389/fnut.2022.1031502

WHO. (2017). In *Guideline: Protecting, Promoting and Supporting Breastfeeding in Facilities Providing Maternity and Newborn Services*. https://www.ncbi.nlm.nih.gov/pubmed/29565522

WHO. (2023). *Breastfeeding*. https://www.who.int/health-topics/breastfeeding#tab=tab_1

Wicinski, M., Sawicka, E., Gebalski, J., Kubiak, K., & Malinowski, B. (2020). Human Milk Oligosaccharides: Health Benefits, Potential Applications in Infant Formulas, and Pharmacology. *Nutrients*, *12*(1). https://doi.org/10.3390/nu12010266

Wong, H. O., Fung, H., & Rogers, M. S. (1997). Dietary patterns amongst ethnic Chinese pregnant women in Hong Kong. *J Obstet Gynaecol Res*, *23*(1), 91-96. https://doi.org/10.1111/j.1447-0756.1997.tb00812.x

Wong, V. W., Ng, Y. F., Chan, S. M., Su, Y. X., Kwok, K. W., Chan, H. M., Cheung, C. L., Lee, H. W., Pak, W. Y., Li, S. Y., & Wong, M. S. (2019). Positive relationship between consumption of specific fish type and n-3 PUFA in milk of Hong Kong lactating mothers. *Br J Nutr*, *121*(12), 1431-1440. https://doi.org/10.1017/S0007114519000801

Woo, J., Leung, S. S. F., Ho, S. C., Lam, T. H., & Janus, E. D. (1997). A food frequency questionnaire for use in the Chinese population in Hong Kong: Description and examination of validity. *Nutrition Research*, *17*(11-12), 1633-1641. https://doi.org/https://doi.org/10.1016/S0271-5317(97)00170-X.

Wu, L. D., Ruhaak, L. R., & Lebrilla, C. B. (2017). Analysis of Milk Oligosaccharides by Mass Spectrometry. *Methods Mol Biol*, *1503*, 121-129. https://doi.org/10.1007/978-1-4939-6493-2_10

Wu, S., Grimm, R., German, J. B., & Lebrilla, C. B. (2011). Annotation and structural analysis of sialylated human milk oligosaccharides. *J Proteome Res*, *10*(2), 856-868. https://doi.org/10.1021/pr101006u

WWF. (2020). *Ecological footprint*. https://d3q9070b7kewus.cloudfront.net/downloads/green_cities_ecological_footprint.pdf

Xu, G., Davis, J. C., Goonatilleke, E., Smilowitz, J. T., German, J. B., & Lebrilla, C. B. (2017). Absolute Quantitation of Human Milk Oligosaccharides Reveals Phenotypic Variations during Lactation. *J Nutr*, *147*(1), 117-124. https://doi.org/10.3945/jn.116.238279

Yang, R., Gao, R., Cui, S., Zhong, H., Zhang, X., Chen, Y., Wang, J., & Qin, H. (2019). Dynamic signatures of gut microbiota and influences of delivery and feeding modes during the first 6 months of life. *Physiol Genomics*, *51*(8), 368-378. https://doi.org/10.1152/physiolgenomics.00026.2019

Yassour, M., Jason, E., Hogstrom, L. J., Arthur, T. D., Tripathi, S., Siljander, H., Selvenius, J., Oikarinen, S., Hyoty, H., Virtanen, S. M., Ilonen, J., Ferretti, P., Pasolli, E., Tett, A., Asnicar, F., Segata, N., Vlamakis, H., Lander, E. S., Huttenhower, C., . . . Xavier, R. J. (2018). Strain-Level Analysis of Mother-to-Child Bacterial Transmission during the First Few Months of Life. *Cell Host Microbe*, *24*(1), 146-154 e144. https://doi.org/10.1016/j.chom.2018.06.007

Yu, Z. T., Chen, C., & Newburg, D. S. (2013). Utilization of major fucosylated and sialylated human milk oligosaccharides by isolated human gut microbes. *Glycobiology*, *23*(11), 1281-1292. https://doi.org/10.1093/glycob/cwt065

Zahangir, M. S., Hasan, M. M., Richardson, A., & Tabassum, S. (2017). Malnutrition and non-communicable diseases among Bangladeshi women: an urban-rural comparison. *Nutr Diabetes*, *7*(3), e250. https://doi.org/10.1038/nutd.2017.2

Zhang, X., Gong, Y., Chen, Y., Corte, K. D., Duan, R., Tian, G., Liang, Y., Xue, H., Zhang, J., Li, M., He, F., Yang, D., Zhou, R., & Cheng, G. (2020). Nutrition in pregnancy and growth in Southwest China (NPGSC) cohort: Design, implementation, and characteristics. *Paediatr Perinat Epidemiol*, *34*(6), 724-733. https://doi.org/10.1111/ppe.12704

Zhu, L., Luo, F., Hu, W., Han, Y., Wang, Y., Zheng, H., Guo, X., & Qin, J. (2018). Bacterial Communities in the Womb During Healthy Pregnancy. *Front Microbiol*, *9*, 2163. https://doi.org/10.3389/fmicb.2018.02163

Zhu, Y., Luo, G., Wan, L., Meng, J., Lee, S. Y., & Mu, W. (2022). Physiological effects, biosynthesis, and derivatization of key human milk tetrasaccharides, lacto-N-tetraose, and lacto-N-neotetraose. *Crit Rev Biotechnol*, *42*(4), 578-596. https://doi.org/10.1080/07388551.2021.1944973