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DETERMINATION OF CARBOHYDRATE COMPOSITION IN BREAST MILK AND ITS ASSOCIATION WITH INFANT'S GROWTH AND BEHAVIOUR

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BY

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A project submitted as a partial fulfilment of the requirement for the degree of Bachelor of Science (Nutrition and Community Health) from the Faculty of Medicine and Health Sciences, Universiti Putra Malaysia

This project entitled "Determination of carbohydrate composition in breast milk and its association with infant's growth and behaviour" was prepared by Nuruljannah Binti Mohamad Nasri and submitted to the Faculty of Medicine and Health Sciences as a partial fulfilment of the requirement for the degree of Bachelor of Science (Nutrition and Community Health) from the Faculty of Medicine and Health Science, Universiti Putra Malaysia.

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LIST OF ABBREVIATIONS

BAZ	BMI-for-age
BEBQ	Baby Eating Behaviour Questionnaire
BMI	Body Mass Index
FR	Food Responsiveness
GA	General Appetite
HAZ	Height-for-age
HMA	Human Milk Analyzer
HPLC	High-Performance Liquid Chromatography
NHMS	National Health Morbidity Survey
PABA	P-aminobenzoic Acid
SE	Slowness in Eating
SR	Satiety Responsiveness
UNICEF	United Nation's Children's Fund
WAZ	Weight-for-age
WHO	World Health Organization

Abstract

DETERMINATION OF CARBOHYDRATE COMPOSITION IN BREAST MILK AND ITS ASSOCIATION WITH INFANT'S GROWTH AND BEHAVIOUR

Nuruljannah Binti Mohamad Nasri

Breastfeeding is the gold standard of infant feeding, and it is very exclusive as its composition varies within a feed, diurnally and over lactation period. Carbohydrate is the major macronutrient found in breast milk, which comprises lactose and oligosaccharides, and has been suggested to be associated with infant health outcomes. Thus, this study aims to determine the breast milk carbohydrate composition and its association with infant's growth and behaviour. A longitudinal observational study involving 64 infant-mothers dyads was conducted in Klang-Valley. Home visits were done at 2, 4-6 and 8-12 weeks of infant's age. Infant's weight and height were measured, and body weight status was determined. Infant's appetite and behaviour were assessed using the Baby-Eating-Behaviour Questionnaire and 3-Day Infant Behaviour Diary, respectively. Breast milk was collected for fore- and hind-milk at all home visits. Total carbohydrate was determined using human milk analyzer, whereas lactose was determined using high-performance liquid chromatography. The mean weight-for-age and BMI-for-age at week 8-12 were -0.50±0.87 and -0.99±1.01, respectively, indicating a normal growth pattern. Breast milk contains an average of 7.08 ± 0.31g/dL and 7.15 ± 0.20g/dL for total carbohydrate and lactose, respectively, showing an expected level based on literature. Carbohydrate composition was stable across different time-points and within feeding ($p>0.05$). Total milk carbohydrate was positively correlated with infant weight at week 8-12 ($r=0.314$, $p<0.05$), but no significant association was found for lactose per say. No association was found between carbohydrate composition and infant outcomes e.g. appetite, feeding and sleeping behaviour. In conclusion, total carbohydrate was associated with infant weight at later time point of the study, but no association was found with other variables. As milk contains various nutrient and bioactive factors, future research with larger sample size shall explore the potential roles of milk composition for infant's well-being.

Abstrak

PENENTUAN KOMPOSISI KARBOHIDRAT DALAM SUSU IBU DAN PERKAITANNYA DENGAN PERTUMBUHAN DAN TINGKAH LAKU BAYI

Nuruljannah Binti Mohamad Nasri

Penyusuan susu ibu adalah yang paling terbaik bagi bayi dan ianya sangat ekslusif kerana komposisi susu ibu berlainan diantara penyususan, sepanjang hari dan juga sepanjang tempoh penyusuan. Karbohidrat merupakan makronutrien utama dalam susu ibu yang terdiri daripada laktosa dan oligosakarida, serta telah ditemui mempunyai perkaitan dengan perkembangan bayi. Oleh itu, kajian ini dijalankan bagi mengenal pasti komposisi karbohidrat dalam susu ibu serta mengkaji perkaitannya dengan pertumbuhan serta tingkah laku bayi. Satu kajian pemerhatian longitudinal melibatkan 64 pasangan ibu-anak telah dijalankan di Lembah Klang. Lawatan ke rumah telah dijalankan pada 3 waktu mengikut usia bayi iaitu 2, 4-6 minggu serta 8-12 minggu. Berat serta panjang bayi telah diukur bagi mengenalpasti status berat badan bayi. Selera makan serta tingkah laku bayi juga telah diakses menggunakan Baby Eating Behaviour Questionnaire (BEBQ) dan 3-Day Infant Behaviour Diary. Sample susu ibu telah diambil pada sebelum dan selepas menyusu pada setiap kali lawatan ke rumah. Kandungan jumlah karbohidrat telah dikenalpasti menggunakan penganalisis susu ibu MIRIS manakala kandungan laktosa telah dikenalpasti menggunakan *High Performance Liquid Chromatography* (HPLC)Pada minggu 8-12, purata Berat-untuk-umur (WAZ) bayi adalah -0.50±0.87 manakala purata BMI-untuk-umur (BAZ) bayi pula adalah -0.99±1.01, menunjukkan pertumbuhan bayi yang normal. Susu ibu mempunyai purata 7.08 ± 0.31g/dL kandungan laktosa serta 7.15 ± 0.20g/dL kandungan jumlah total karbohidrat, menunjukkan anggaran nilai yang sama ditunjukkan dalam kajian-kajian sebelum ini. Kandungan jumlah karbohdrat dan laktosa adalah stabil pada titik masa yang berbeza dan juga antara sebelum dan selepas menyusu ($p>0.05$). Perkaitan positif telah ditemui antara jumlah karbohidrat dengan berat bayi pada minggu ke 8-12 ($r=0.314$, $p<0.05$), Tetapi perkaitan tidak ditemui untuk kandungan laktosa. Tida perkaitan ditemui antara komposisi karbohidrat dengan pertumbuhan, selera serta tingkah laku pemakanan dan tidur bayi. Kesimpulannya, jumlah karbohidrat mempunyai perkaitan dengan berat bayi pada titik masa yang lanjut, tetapi tiada perkaitan ditemui antara pemboleh ubah yang lain. Disebabkan susu mempunyai pelbagai nutrien serta faktor biokaktof, kajian lanjut dengan saiz sample yang banyak adalah perlu bagi mengkaji potensi peranan komposisi susu untuk kesejahteraan bayi.

CHAPTER 1

INTRODUCTION

1.1 Background

World Health Organization (WHO) recommends mothers to practice exclusive breastfeeding up to 6 months and continue to breastfeed up to 2 years of age or beyond. In Malaysia, the prevalence of exclusive breastfeeding for six months is 47.1% in 2016 (NHMS III, 2016). WHO recommended exclusive breastfeeding as early infant nutrition is a critical period for infant's development. Breastfeeding helps to protect infants from diarrhoea and respiratory infection in the short-term (UNICEF, 2013). Breastfeeding also benefits both mother and infants in the long-term. Infants who are breastfed for a longer period have low morbidity and mortality risk while mothers can gain benefit through prevention of breast and ovarian cancer (Victora et al., 2016). Breastfeeding is the best way for mothers to provide various essential nutrients needed by infants.

Human breast milk is very exclusive that its composition is variable within feeds, diurnally, over lactation and between mothers and population. The composition varies to meet the infant's needs for growth, development and survival (Ballard & Morrow, 2013). There are 3 stages in human breast milk which are colostrum, transitional and mature milk. Every stage of breast milk has different composition according to the time point. Besides that, the compositions are also different between hindmilk and foremilk which hindmilk is more energy-dense, while foremilk is more diluted.

The major macronutrients compositions in breastmilk are carbohydrate, protein and fat. The approximate concentration of macronutrients in mature-term milk are 0.9 to 1.2 g/dL for protein, 3.2 to 3.6 g/dL of fat and 6.7 to 7.8 g/dL of lactose in the first year of lactation (Czosnykowska-łukacka et al., 2018). From the data, the concentration of carbohydrate in human breast milk is the highest among other macronutrients.

There are various types of carbohydrate present in breast milk. The primary carbohydrate of human breast milk is the disaccharide lactose (Eriksen et al., 2018). Lactose is the major disaccharide carbohydrate in human breast which is approximately 70-83% of total carbohydrates. It provides 40% of the total calories of human milk (Léké et al., 2019). Lactose is important for the development of human brain and gut physiology, for instance, prebiotic effects, softening of stools, and enhancement of water, sodium and calcium absorption (Chang et al., 2015). Lactose also aids the absorption of minerals and calcium for the development of infants (C. R. Martin, Ling, & Blackburn, 2016). Besides that, a stable concentration of lactose is crucial in maintaining constant osmotic pressure in human milk.

Lactose composition in human milk was found to be positively associated with infant growth, demonstrating a change in infant weight, adiposity and BMI between 3 and 12 months of age (Prentice et al., 2016). Based on the study, the author proposed that higher intake of lactose in human breast milk could promote storage of glycogen and fat which in that way promoting growth. Another study found that human milk lactose concentration (average of 2 weeks and 4 months values) was positively associated with infant weight-for-length z-score up to 6 months of age in the small cohort. However, this association was found only in infants of overweight or obese mothers (Young et al., 2017).

Apart from growth, carbohydrates in human breast milk were also associated with appetite and feeding behaviour of infants. The milk intake depends on infant's demand and is determined by gastric emptying which is the key regulator that leads to earlier hunger sensation (Khan et al., 2013). A study also found that lactose feeds are associated with gastric emptying (Woolridge & Fisher, 1988). Aside from lactose, oligosaccharide also showed faster gastric emptying that leads to hunger (Indrio et al., 2009). The feeding behaviour of infants is also associated with carbohydrate composition in breast milk. Studies show that there was a positive association between lactose concentration and milk intake per day (Arthur, 1989). This statement was supported by a recent study by Khan et al that shows the lactose concentration in breastmilk was associated with breast milk production and frequency of feeding. From the findings, it was shown that lactose is associated with appetite and behaviour of infants, however, there is no study reported on the association of oligosaccharide with appetite and behaviour. Therefore, it is important to study the association of oligosaccharide with appetite and behaviour of infants as it showed faster gastric emptying that lead to the regulation of appetite and behaviour.

1.2 Problem Statements

World Health Organization (WHO) reported that the number of overweight children below the age of five is estimated to be over 41 million and almost half of them lived in Asia including Malaysia. The alarming prevalence of childhood obesity has become serious public health challenges of the 21st century. Several risk factors that are associated with childhood obesity including early infant nutrition. Early postnatal nutrition is crucial for optimal infancy growth and is associated with long-term health outcomes. Breastfeeding has been associated with slower gains in infancy weight and body fat (Prentice et al., 2016). A study found that rapid early infant weight gain predisposes to an adverse metabolic phenotype in later life with increased risk of overweight, central adiposity and insulin resistance (Prentice et al., 2016). A recent finding from a study found that breastfeeding is associated with lower rates of infancy weight and length gain after the age of 2 months (Eriksen et al., 2018). Nevertheless, these studies do not consider the components of breast milk that are associated with the growth of infants. A recent study on human milk oligosaccharide (HMOs) found that HMOs was associated with excessive weight gain among exclusively breastfed infants (Larsson et al., 2019). This shows that there is contradictory in the association between breastfeeding and optimal growth. There is also the possibility that some mothers produced certain HMOs profile or infants with certain microbiome profiles may possibly link with early infant growth, however these are largely unexplored.

Infant's appetite and behaviour are also associated with the long-term effects on health. Vigorous milk-feeding style at 2-4 weeks of age which is characterized by long bursts of rapid, high pressure of sucking with shorter resting period was found to be associated with higher adiposity 2 years later (Agras et al., 1990). This finding was supported by another study which found that infants who have a more avid sucking style has higher a risk to be obese (Stunkard et al., 2004). From the findings, it is important to study the association of carbohydrate composition with appetite and behaviour as these components will eventually lead to adiposity and obesity in later life.

In Malaysia, infant formula possesses rapid sale in the market as there was an increase of 43% sales from 2008 until 2013 (Baker et al., 2016). It is well established that formula milk was created to be as close as possible to human milk. However, both kind of milks differ in terms of both nutritional and biological constituents. It is because some formulas contain added sugars that are not present in breastmilk and the actual sugar content, in terms of both type and proportion, of infant formula is not widely known (Walker & Goran, 2015). A study found that lactose was detected in all formula milk, except for lactose-free infant formula milk for lactase-deficient infants, which contained maltose and glucose (Ferreira, Gomes, & Ferreira, 1998). Another recent study conducted in Saudi Arabia found that lactose is included in all formulas and one formula brand has included glucose, galactose, maltose and polysaccharide in the formula (Almazrooy et al., 2017). This shows that the composition in formula milk varies for the types of carbohydrates incorporated in the milk. Another concern is the high composition of sugar in formula milk. It was reported that 80% of formula products marketed for young children contain high sugar amount, including corn syrup and sucrose which is not the major carbohydrate found in breast milk (Palafox & Harris, 2017). The higher intake of added sugar will eventually increase the adiposity level in infants (Amarra, Khor, & Chan, 2016).

Based on Standard for Infant Formula and Formulas for Special Purposes Intended for Infants developed by Food and Agriculture Organization of the United Nations and World Health Organization, the nutritional composition of formula milk should follow the minimum and maximum level of carbohydrate. In this guideline, lactose and glucose should be the preferred carbohydrates in formula milk (*Standard for Infant Formula and Formulas*, 2015). From this guideline, the concentration of lactose is general, which is not specific to the time-point. Besides that, there is no specific guideline for lactose and oligosaccharides concentration in the formula milk. Thus, many studies should be done to improve the composition of formula milk to be as close as possible to human breast milk. Although breastmilk is the best milk for the infants, formula milk is the second option after breastmilk or donor milk due to certain circumstances which makes the formula milk is high in demand in the formula milk market.

Moreover, there is an increase in the development of awareness and knowledge in the study of human breast milk composition. Determination of human breast milk composition has been a topic of extensive research around the world among various populations and ethnic groups (Chang et al., 2015). However, only a few reports have been published in Malaysia on the composition of human breast milk. There was no study which aims to determine the carbohydrate composition across different time-point was conducted in Malaysia. Moreover, the study on association of carbohydrate composition with infant's growth are extremely limited (Gridneva et al., 2019). Apart from that, the association of carbohydrate composition with infant's behaviour also limited as most studies relate the association of feeding behaviour with milk supply (Khan et al., 2013). As the composition of human breast milk varies among the different population, it is beneficial to study the carbohydrate composition among lactating women in Malaysia.

1.3 Research Questions

1. What is the concentration of total carbohydrate and lactose in breast milk across different time point (week 2, week 4-6 and week 8-12)?
2. What is the trend of changes of carbohydrate concentration of breast milk across time point (week 2, week 4-6 and week 8-12) within feeding?
3. Is there any association of carbohydrate composition with growth, appetite and feeding behaviour of infants?

1.4 Significance of Study

This study is conducted to investigate the association of carbohydrate composition with the growth and behaviour of infants. The carbohydrate composition is determined to see the changes in the trend of total carbohydrate, lactose and oligosaccharide composition across different time point. The association of carbohydrate composition with growth and behaviour is determined across different time-point. Thus, the findings of this study can fill the gap in knowledge and understanding of human breast milk composition. The information can help researchers to have a better understanding on how different composition of carbohydrate across different time point is associated with the growth, appetite and feeding behaviour of infants.

The findings of this study can provide baseline data for extended research on human breast milk composition. The findings of this study can be used by researchers to better understand other breast milk composition that changes across different time-point. In addition, the enhancement of knowledge and a better understanding of human breast milk composition would be useful to understand factors that are associated with infant growth and development as early nutrition is crucial for infants.

Moreover, this study can be used by policymaker and manufacturers to revise the nutrition composition of formula milk. As infant formula is intended as an effective substitute for infant feeding, many efforts have been made to imitate the nutritional composition of breast milk (C. R. Martin et al., 2016). This information may be useful for the incorporation of oligosaccharide in milk formulas (Cavalli, Teng, Battaglia, & Bevilacqua, 2006). Recently updated FDA (Food and Drug Administration) rule on current Good Manufacturing Practices for infant formula, 21 CFR 106.96 [6], requires that formula milk satisfy the quality factors of normal physical growth and biological quality of protein component. Federal and local agencies of different countries control and monitor infant formula regulations according to WHO guidelines. This guideline includes the requirement for quality and manufacturing practices in the countries. Therefore, any addition and improvement of guidelines regarding nutritional composition would benefit federal and local agencies in different countries around the world. Besides that, it would be beneficial for manufacturers to improve their products to be as close as possible to human breast milk.

1.5 Objectives

1.5.1 General Objective

To study the association of carbohydrate composition in breast milk with growth and behaviour of infants.

1.5.2 Specific Objectives

1. To determine the trend of changes of total carbohydrate in breast milk across 3 different time-points (week 2, Week 4-6 and week 8-12) within feeding.
2. To determine the trend of changes of lactose in breast milk across 2 different time-points (week 2 and week 8-12) within feeding.
3. To assess the growth (weight-for-age, length-for-age and BMI-for-age) of infants at week 8-12.
4. To assess the behaviour (appetite, duration and frequency of feeding and duration of sleeping) of infants at week 4-6.
5. To determine the association of carbohydrate composition in breast milk with growth and behaviour of infants.

1.5.3 Null Hypothesis

1. There is no association of carbohydrate composition in breast milk at end time point with growth and behaviour of infants.

1.6 Research Conceptual Framework

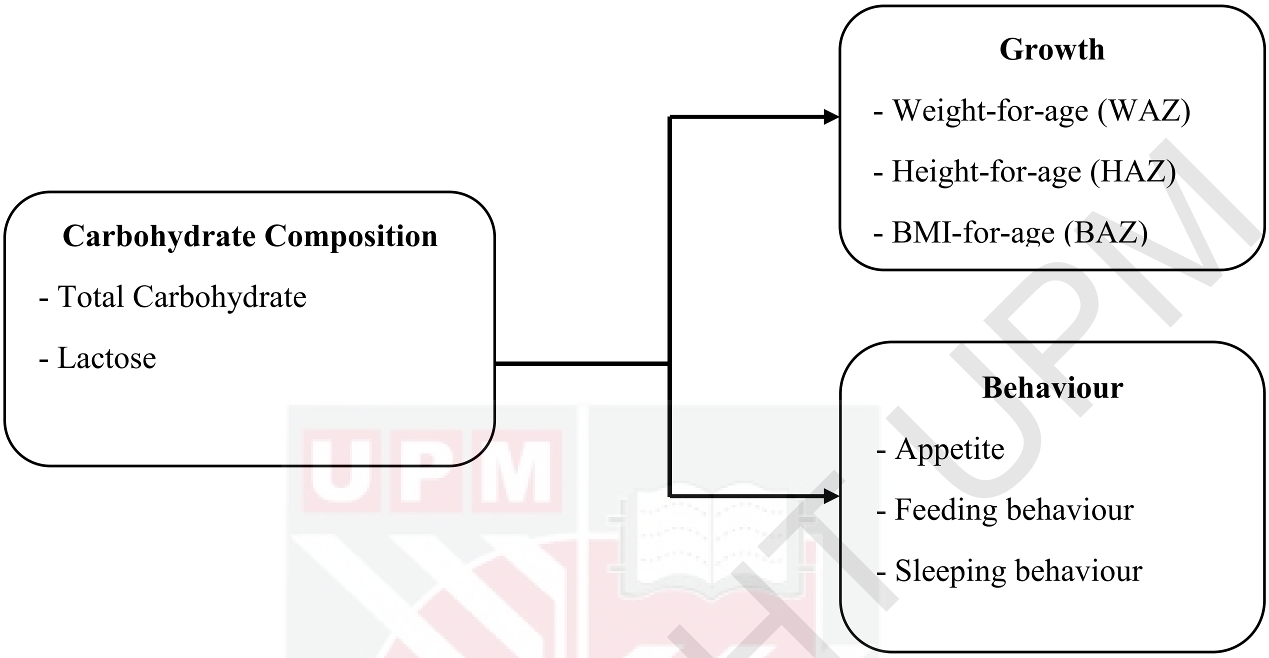

Figure 1: Research conceptual framework

Figure 1 shows the conceptual framework of this study. This conceptual framework represents the association between carbohydrate composition in human breast milk with growth and behaviours of infants. The independent variable in this study is the carbohydrate composition across different time point (week 2, week 4-6 and week 12-16), which are total carbohydrate and lactose. The dependent variables are growth (Weight-for-age, Length-for-age and BMI-for-age) and behaviours (appetite, duration and frequency of feeding and duration of sleeping) of infants.

CHAPTER 2
LITERATURE REVIEW

2.1 Importance of early nutrition for infant health

Infant programming has been a current interest in the research to improve the health quality of infants in the long term. Programming can be defined as “a stimulus or insult during critical or sensitive period of development that gives long-term or lifetime effects on an organism”(Lucas, 2005). Infant programming is important as early nutrition is crucial for development of infants as it is the phase for rapid growth. It is also important as it can give long term health effect in adulthood (Lanigan & Singhal, 2009).

A large-scale collaborative project has been conducted from 2012 to 2017 on EarlyNutrition in twelve European countries, the USA and Australia (Berthold et al., 2012). This project was an integrated work that addresses 4 main themes, which were mechanisms of early nutrition programming effects, observational studies of determinants in contemporary cohorts, human intervention studies on modifiable determinants and strategic integration and recommendation development which complemented by dissemination and training activities. It was a combination of animal, observational and human intervention studies that seek evidences to help formulate recommendations. This project has contributed a lot of knowledge and convincing evidence on early nutrition and health outcomes. This shows that early nutrition is a crucial period that needs a lot of attention and special care for the development and health of infants.

Several animal studies have shown that nutrition in early life can influence the outcomes of blood lipid, blood pressure, adiposity, atherosclerosis, behaviour, learning and longevity later in adulthood (Lucas, 2005). Human studies were also conducted to study the association of early nutrition with health outcomes. It was found that infants who are breastfed for a longer period have lower mortality and morbidity risk (Victora et al., 2016). A study by Ekelund et al., in 2007 found that faster weight gain in the first 6 months of life are independently associated with a clustered metabolic score (waist circumference, glucose and insulin concentrations, fasting TAG, HDL cholesterol and blood pressure) in adolescents (Ekelund et al., 2007). This finding supports the hypothesis that early rapid growth increases risk of CVD in later life (Ekelund et al., 2007). Another study reported that rapid weight gain in the first week of life has been associated with a 30% increased the risk of being overweight in adulthood (Plagemann, 2005). These findings prove that early nutrition in infant's life plays an important role for the determinant of health status in later life.

2.2 Breastfeeding and milk composition

World Health Organization (WHO) recommends mothers to practice exclusive breastfeeding for 6 months and continue to breastfeed up to 2 years of age or beyond alongside complementary feeding. This recommendation is supported by Ministry of Health Malaysia (MOH) through the implementation of breastfeeding policy and Baby Friendly Hospital Initiative (BFHI). Various efforts have been made to enhance breastfeeding efficacy among mothers by sharing knowledge and giving support to breastfeeding. Globally, less than 2/5 infants are exclusively breastfed for 6 months of life based on WHO Data in 2001. In Malaysia, the prevalence of exclusive breastfeeding for 6 months recorded in National Health and Morbidity Survey III in 2016 was only 47.1%. This shows that the prevalence of infants who are breastfed exclusively in low in global as well as in Malaysia.

Breastfeeding is a gold standard feeding, which is the most effective way for mothers to deliver essential nutrients needed for infants. Breastfeeding delivers many health benefits for infants in both short and long term. In term of short-term benefits, a finding from a study showed that breastfeeding is helpful to reduce paediatric infections, immune disorders and death in the first year of life (Jackson & Nazar, 2006). Besides that, breastfeeding was also found to be correlated with mother-infant relationship. A study was conducted to compare mother-infant relationship between breastfed and bottle-fed infant. The study found that infants who are breastfed display more positive interaction with mothers during feeding through touching (Kuzela, Stifter, & Worobey, 1990). Finding from this study also showed that breastfed infants demonstrated more irritability compared to bottle-fed infant, which suggest that the irritability is a positive act to promote more interaction between mother and infant .

The long-term benefits play a role in the public health area. Breastfeeding was found to be associated with cognitive development. A 3-decade prospective birth cohort study conducted in Brazil found that infants who were breastfed for 12 months or more have higher IQ score 30 years later, suggesting that breast milk plays a role in cognitive development for long-term effect (Victora et al., 2015). This finding is supported by a recent cohort study conducted among Asian infants, which aims to study the association between milk feeding and cognition. It was found that nutrients in breast milk may improve child's cognition while children who were breastfed directly from the breast have better memory (Pang et al., 2019). Another study that aims to study on the association between breastfeeding duration and cognition among Korean infants found that breastfeeding for longer than 9 months improved cognitive development of Korean infants (Lee et al., 2016).

Apart from that, breastfeeding was also found to be associated with protection against the development of type 2 diabetes mellitus. A systematic review and meta-analysis which aim to study the long-term consequences of breastfeeding on cholesterol, obesity, systolic blood pressure and type 2 diabetes found that breastfeeding decreased the odds of type 2 diabetes. There was no association found for total cholesterol and blood pressure (Horta, Loret De Mola, & Victora, 2015). In addition to this, a longitudinal study conducted in Australia found that infants who were breastfed for more than 4 months show protective effect against the development of type 2 diabetes in young adulthood (Mamun et al., 2015).

Breastfeeding also benefits the mother's health in long-term health effects. A meta-analysis on breastfeeding and ovarian cancer found that ever breastfeeding and longer duration of breastfeeding lower epithelial ovarian cancer risk, which is one of top cancer among women (Musselwhite, 2013). Besides that, mothers who breastfed their infants benefits from protection against breast cancer. A case-control study conducted in Germany showed that prolonged breastfeeding might act as a protective role against the development of breast cancer in predominantly premenopausal women (Chang-claude et al., 2011). Moreover, breastfeeding may also help to prevent mothers with gestational diabetes mellitus from the development of type 2 diabetes mellitus after delivery. This statement is supported by a cohort study conducted among pregnant women with GDM. The finding of this study showed that longer duration of breastfeeding is associated with lower 2 years incidences of diabetes mellitus after delivery (Gunderson et al., 2016). This evidence proved and strengthen the reason why WHO recommends mothers to exclusively breastfeed their infants for 6 months and continue up to 2 years as studies show that longer duration of breastfeeding may protect from various non-communicable diseases, which can be prevented. Therefore, knowledge and understanding of this health issue are very important as it may improve mother's efficacy and stand to breastfeed their infants.

Breast milk is very exclusive that its composition varies within feeds, diurnally, over lactation and between mothers and population (Ballard & Morrow, 2013). The variability of the composition is due to demands of infants for their growth, development and survival. Generally, there are 3 stages in human milk which the compositions and concentrations vary over lactation period. The stages are colostrum, transitional and mature milk. A study of human milk composition by

Ballard and Morrow in 2013 revealed that colostrum is the first fluid produced by mothers after delivery and it remains for a few days. It is distinct in volume, appearance and composition, which is low in quantity and rich in immunologic components such as secretory immunoglobulin (IgA), lactoferrin, leukocytes and developmental factors such as epidermal growth factor (EGF). The nutritional composition is distinct which it contains a relatively low concentration of lactose and fat, whereas the concentration of whey protein is high. Based on the data, the composition of colostrum indicates that the primary role of colostrum is immunologic rather than nutritional, which functions to protect the baby as it emerges from sterile conditions in the womb to exposure of environmental conditions and pathogens. Moreover, colostrum also appears to promote growth as it contains many growth factors in greater concentration than in mature milk (Andreas et al., 2015). The second stage is transitional milk, which typically occurs from 5 days to 2 weeks of the postpartum period. In this stage, the composition of breast milk shares some of the characteristics of colostrum but there is also elevation of nutritional and developmental needs for rapidly growing infants (Ballard & Morrow, 2013). The last stage of human breast milk is mature milk, which considered to be fully matured by week 4 to 6. In this stage, the nutritional composition remains relatively similar in composition, even though subtle changes in milk concentrations do occur throughout lactation.

Apart from that, breast milk concentrations also vary within a feed. Foremilk refers to the milk in the beginning of feeding whereas hind milk refers to milk produced at the end of a feeding. It was found that hindmilk contains higher energy, fat, vitamin A and E compared to foremilk (Nielsen et al., 2017). Moreover, a study from Finland found that hindmilk contains 2-3 times fat content compared to foremilk (Saarela et al., 2005). Another study on changes of milk between foremilk and hindmilk also found that hindmilk is more energy dense as it contains higher lipid content to ensure that infant satiety and energy needs for growth are well met. Hindmilk has been successfully used to improve growth of very premature infants and is recommended for their nutritional management (Ballard & Morrow, 2013). This study also revealed that triglyceride level is higher in hindmilk compared to foremilk, suggesting that it is important for self-control during breastfeeding.

2.3 Macronutrients composition in breast milk

The composition of breast milk has been reported in many studies from diverse population. The major macronutrients in breast milk are protein, fat and carbohydrate. A recent study was conducted in 2018 to study the composition of breast milk and the data showed that mature-term milk contains approximately 0.9 to 1.2 g/dL of protein, 6.7 to 7.8 g/dL of lactose and 3.2 to 3.6 g/dL of fat (Czosnykowska-łukacka et al., 2018). The energy content was estimated to be 65 to 70 kcal/dL and highly correlated with the fat content in milk. In this study, it was found that the concentration of protein and fat increase over prolonged lactation up to 2 years. However, the concentration of carbohydrates was found to be significantly decreased.

Another study conducted among 436 healthy lactating mothers in China showed huge individual variations of milk composition. This study also aimed to determine the changes of concentration over lactation period for 5 consecutive time point, starting from day 5 to day 240 of lactation after birth. The average concentration of mature milk was 61.3 kcal/dl for total energy, 7.1 g/dl for lactose, 0.9 g/dl for protein and 3.4 g/dl for fat (Yang et al., 2014). In terms of changes of macronutrients composition across lactation, it was found that protein concentration reduced gradually and maintained in 61-240 days whereas fat concentration did not change significantly after 12-30 days. Lactose concentration was found to be significantly higher in 12-30 days and maintained stable after that.

In addition, another breast milk macronutrients composition study across lactation period conducted among 2632 healthy lactating women found that the macronutrient composition of breast milk per 100 mL of mature breast milk was 1.4 g for protein, 7.1 g for lactose and 3.0 g for fat whereas the energy content was 61.1 kcal (Chang et al., 2015). From this study, it was also found that the protein concentration decreases gradually over lactation period, which the concentration was 2.2 g/dL in 0-1 week, then reduce slightly on week 1-2 (2.0 g/dL) and continue to reduce on month 2-3 (1.4 g/dL). The concentration is maintained from 3-4 months to 7-8 months (1.3 g/dL). For fat concentration, it was also found that the mean fat levels varied among lactation period (2.7 – 3.2 g/dL). However, there was no significant difference. Apart from protein and fat, lactose concentration was found to be similar throughout lactation period.

From these studies, the concentration of macronutrients are summarized in the table below :

Table 1: Macronutrients in breast milk

(Author/Year)	(Czosynkowska-lukacka et al., 2018)	(Yang et al., 2014)	(Chang et al., 2015)
Protein (g/dL)	0.9 – 1.2	0.9	1.4
Fat (g/dL)	3.2 – 3.6	3.4	3.0
Lactose (g/dL)	6.7 – 7.8	7.1	7.1
Energy (kcal/dL)	65 - 70	61.3	61.1

From the table above, it can be concluded that lactose is the major nutrients found, followed by fat and protein. It was also shown that the concentration is quite similar which there are no huge differences of concentration between studies, suggesting that the breast milk concentrations are almost similar among different population. This may be a proof that these are the range of nutrients needed for infant's development and wellbeing.

Human breast milk contains around 400 different proteins which carry variety of functions (Lönnerdal, 2004). The protein in human milk is divided into whey which is the major fraction (60-80%) and casein which is the smaller fraction of protein. Each complex comprises of specific peptides that make up the fractions (Liao et al., 2011). Whey protein is classified as fast protein based on its characteristic of faster digestion and absorption of amino acid compared to casein (Boirie et al., 1997). The whey fraction comprises of α-lactalbumin, lactoferrin, igS, lysosome and serum albumin whereas casein fraction comprises of α-, β-and κ-casein (Lönnerdal, 2004). Different types of protein fraction carry specific function in the body. The α-lactalbumin is needed for lactose synthesis, which creates an osmotic drag to facilitate milk production and secretion in the mammary gland (K. Brew, 2003). It also binds to cations that may facilitate the absorption of essential minerals needed for growing infants (Lo, Ph, Lien, & Ph, 2003). Lactoferrin in the other hands involved in iron homeostasis, regulation of immune cells functions, immune responses as well as antimicrobial effects (Rai et al., 2014). Therefore, it can be concluded that protein content in breast milk provides various health benefits to infants, namely, to provide nutrition, carry out antimicrobial and immunomodulatory activities as well as stimulate the absorption of nutrients to stimulate growth (Lönnerdal, 2004). A finding from DARLING Study conducted in 1991 found that the concentration of protein in breast milk is not affected by maternal diet. The concentration increases with maternal body weight for height and decreases in mothers that produce higher amount of milk (Nommsen et al., 1991).

Lipid on the other hand contributes the highest source of energy in breast milk which is about 40%-55% (Berthold Koletzko et al., 2001). About 98% of lipid fraction consists of triglyceride, which is the major lipid presents in breast milk. The other lipid fractions are monoacylglycerides, diacylglycerides, cholesterol, free fatty acid and phospholipids (Lopez & Ménard, 2011). There are over 200 fatty acids found in breast milk. However, most of the fatty acids are present in very low concentrations (B. Koletzko, 1988). The major fatty acids found in breast milk are oleic, palmitic and linoleic acid (Martin et al., 1993).

Apart from its function to provide energy, fat in breast milk was also found to be a protective mechanism against microorganism. Both membrane and core components of lipid provide protection against microorganism by different mechanism (Hamosh et al., 1999). This study explains that the major protective membrane glycoproteins, mucin, and lactadherin are resistant to conditions in the infant's stomach and maintain their structure and function even at low pH and in the presence of the proteolytic enzyme pepsin. Besides that, the core triglycerides upon hydrolysis by digestive lipases produce free fatty acids and monoglycerides, which are amphiphilic substances that able to lyse enveloped viruses, bacteria, and protozoa.

The composition of fat in breast milk is highly variable compared to other macronutrients. It is characterized by high contents of palmitic and oleic acid (Ballard & Morrow, 2013). A study on fat content over 24 hours showed that the milk fat content was significantly lower in night and morning compared to milk feeding in the afternoon and evening (Kent et al., 2006). The fatty acid profile of breast milk was associated with maternal diet, specifically long-chain polyunsaturated fatty acids (Martin et al., 2012). Fatty acids secreted in milk are mobilized from adipose tissue stores. It is directly absorbed from maternal dietary lipid (Del Prado et al., 2001).

2.4 Carbohydrate composition in breast milk

Carbohydrate is one of the macronutrients aside from protein and fat. A study on breastmilk composition by Leke at al., in 2019 found that breast milk contains approximately 70-83% of total carbohydrate, that account for 40% of total calories, whereas disaccharide lactose is the major carbohydrate found in breast milk (Eriksen et al., 2018). Milk is regarded as the only known significant source of lactose (Fox, 2009). It is composed of monosaccharide glucose and galactose. Both monosaccharides are linked together by a $\beta 1 \rightarrow 4$ glycosidic bond (Fox, 2009). The concentration of lactose in human milk is the least variable. However, higher concentration of lactose was found in the milk of mothers that producing a high quantity of milk (Nommsen et al., 1991).

Many types of researches were conducted to study the importance of lactose for infants. It is found to be important for the development of the human brain and gut physiology, for instance, prebiotic effects, softening of stools, and enhancement of water, sodium and calcium absorption (Chang et al., 2015). This statement is supported by a study which found that lactose aids the absorption of minerals and calcium for the development of infants (Martin et al., 2016). Another study that compares between lactose containing and lactose free formula also reported that lactose increases the absorption of calcium (Abrams et al., 2002). Besides that, a stable concentration of lactose is crucial in maintaining constant osmotic pressure in human milk (Camilia et al., 2016).

The other carbohydrate's component is human milk oligosaccharides (HMOs) which comprise approximately 1 g/dL in human milk depending on stages of lactation and maternal genetic factors (Ballard & Morrow, 2013). It is an important component of carbohydrate as it is the third largest component found in the milk (Coppa, Pierani, & Giorgi, 1993). There are around 3 to 22 saccharide units per molecule and are made up of 5 different monosaccharides that varies in sequences and orientations which are L-fucose, D-glucose, D-galactose, N-acetylglucosamine and N-acetylneuraminic acid. There are more than 200 different types of oligosaccharides found in breast milk, all of which feature lactose at the reducing end (German et al., 2008).

HMOs is widely known as prebiotics, which stimulates the colonization of beneficial gut microbiota. Recent studies also suggested that HMOs also act as signalling molecules, immune-modulators, and possible brain function developers (Wu et al., 2018). A study also found that HMOs play an important role in preventing neonatal diarrhoeal and respiratory tract infection (Andreas et al., 2015). Thus, HMOs are needed for the promotion of gut microbiota and immune function. Other carbohydrates such as glucose, galactose, sucrose and maltose were not detected in a study of sugar determination of human milk from Korean mothers (Chang et al., 2015).

In a nutshell, stable and adequate concentration of macronutrients in breast milk is crucial as it is needed for physiological function, stability as well as biochemistry of nutrients. Different macronutrients carry different function for development of infants. Moreover, some of nutrients changes according to time, maternal diet or infant's demand, verifying that human breast milk is unique and gold standard for feeding of infants for their development and survival.

2.5 Determination of carbohydrate in breast milk

The carbohydrate in breast milk can be determined for its total carbohydrate, and lactose. Different carbohydrates have a different method for determination according to the accuracy and sensitivity of the instruments.

2.5.1 Total Carbohydrate

Total Carbohydrate is composed of lactose and other carbohydrate compounds in the breast milk. It can be analysed using human milk analyser (HMA), which is designed to measure the macronutrients content in human milk. It is useful to measure fat, protein, lactose and total solids simultaneously. This analyser uses a mid-infrared spectroscopy which has been proven to be a useful analytical method for macronutrient determination in human milk (Michaelsen, 1988.). This method is widely used in determination of macronutrients in breast milk across different nations, for example in Poland, France and Spain [(Czosnykowska-łukacka et al., 2018); (Léké et al., 2019) & (García-Lara et al., 2012)].

A recent study by Giuffrida et al., (2019) was conducted to evaluate the accuracy and precision of HMA by comparing it with the reference method, which is HPAEC-PAD chromatography. This study explained that HMA requires minimal training operator, and promise a higher throughput, relatively less volume of human milk using and can be executed by user who has undergone simple training compared to the reference method. From the analysis of breast milk by these 2 methods, no significant difference was found for the determination of lactose. It shows that HMA is reliable for quantification of lactose (Giuffrida et al., 2019).

Another study on evaluation of mid-infrared analyser also supported that HMA provides efficient and practical method for quantification of macronutrients in breast milk (Casadio et al., 2010).

2.5.2 Lactose

Lactose concentration in breast milk can be determined through various methods which are enzymatic, redox, polarimetry and high-performance liquid chromatography. Enzymatic method is an old method which utilize enzyme that react with substrate. The advantages of this method are rapid sample preparation, high sensitivity, ease of automation and do not require expensive equipment for analysis. However, the enzymes are expensive compared to other chemical reagent and the enzymes can be degraded after a long period of time (White, C., 1991). Besides, redox method is a simple method which titration is performed with an oxidizing agent, but the analysis can be interfered by other biological compounds and temperature is required to be controlled (Sexton & Leann, 2004). The other method is polarimetry method which utilizes the bending of polarized light. This method is quite complicated as it requires mathematical treatment of the measured specific location (Birch, G., 1985). Therefore, this method is not suitable for quantification of lactose as other components can interfere with the results obtained. On the other hand, high performance liquid chromatography (HPLC) is the common method used as it is sensitive, able to separate compounds and applicable to analyse substances that become interests (Meyer et al., 2001). This shows that HPLC is the best method for quantification of lactose.

A study was conducted to develop a sensitive, reliable and selective reversed-phase HPLC method for lactose determination (Sexton & Leann, 2004). In this method, prepared samples are transported using a medium which is known as mobile phase in the form of liquid. Both samples and mobile phase are pushed or forced by pump to move through a column which is the stationary phase. Both mobile and stationary phases play a role for determination of components, therefore specific mobile and stationary phase need to be chosen according to components of interest. This will allow for separation of mixture for determination. Reversed phase HPLC is the most popular chromatography technique that comprises of polar mobile phase with nonpolar stationary phase. Basic HPLC instruments are solvent reservoirs, pump, injector, column and detector. The column used for determination of carbohydrates are C8 and C18 column. The sample can be detected using UV-Vis and diode array detector. The flow rate is set to be 0.5 mL/min. As HPLC often used for separation of mixture, reductive amination method is used to separate the sugar molecules. P-aminobenzoic acid is used to attack reducing end of sugar that results in the ring opening while sodium cyanoborohydride will be needed to break the double bond between carbon and nitrogen for the final product.

HPLC method is used in many studies on concentration of lactose in milk [(Ferreira et al., 1998) & (Meyer et al., 2001)]. This shows that this is the common method used in many studies and reliable results can be obtained.

2.6 Association of carbohydrate with growth of infants

The lactose composition in human milk was found to be positively associated with growth of infants in few studies. A study was conducted to see the association of breast milk content with infancy growth, which hindmilk samples were collected from 614 mothers in the cohort study where the infant's anthropometry was measured. The finding of this study shows that lactose composition can affect change in infant weight, adiposity and BMI between 3 and 12 months of age. The authors proposed that higher intake of lactose in human breast milk could promote storage of glycogen and fat which in that way promoting growth (Prentice et al., 2016). A recent study that aimed to see the correlation of carbohydrate in milk with body composition of infants found that human milk lactose concentration (average of 2 weeks and 4 months values) was positively associated with infant weight-for-length z-score up to 6 months of age in the small cohort. However, this association is found only in infants of overweight or obese mothers (Gridneva et al., 2019). The finding of this study also revealed that high lactose calculated daily intake was associated with higher fat mass, fat mass index, fat mass percentage, ratio of fat mass per fat free mass and lower fat-free mass index. Despite of these findings, there were only a few studies reported on lactose concentration in breast milk and infant's growth.

Apart from lactose, oligosaccharide also plays an important role for the growth of infants. A recent study on human milk oligosaccharide found that different oligosaccharide affects the growth at different time-point (Alderete et al., 2015). Total human milk oligosaccharide (HMOs) , HMO-bound fucose as well as 2′-fucosyllactose (2′-FL) at 5 months were positively associated with fat mass index and weight velocity from 0 to 5 months whereas lacto-N-neotraose was negatively associated with height-for-age Z-score and weight velocity from 0 to 5 months. The possible mechanism that link human milk oligosaccharides with growth of early infancy is due to its mechanism in the development of infant microbiome (Wang et al., 2015). HMOs is not degraded by infant digestive system. It reaches distal parts of the intestine to be metabolized by gut microbiota. Then, the gut microbiota plays a role to harvest and store energy for the host. Studies have shown that specific microbiome pattern can have an increased ability to harvest energy from the diet and thus accelerate growth of infants (Larsson et al., 2019). This shows that specific types of oligosaccharide are associated with growth of infants.

2.7 Association of carbohydrate with behaviour of infants

The composition of breast milk may influence infant's feeding pattern and appetite. The macronutrients and milk volume affect gastric emptying of infants. The milk intake depends on infant's demand and is determined by gastric emptying which is the key regulator that leads to earlier hunger sensation (Khan et al., 2013). A study found that lactose feeds were associated with gastric emptying. It is because high lactose indicates low-fat feeds that result in gastric emptying which require frequent feeding. This condition will eventually stimulate the milk production to meet the infant's demand (Woolridge & Fisher, 1988). Aside from lactose, oligosaccharides also showed faster gastric emptying that leads to hunger (Indrio et al., 2009). The feeding behaviour of infants is also associated with carbohydrate composition in breast milk. Findings from a study show that there is a positive association between lactose concentration and milk intake per day (Arthur, 1989). This statement is supported by a recent study by Khan et al that shows that the lactose concentration in breastmilk is associated with breast milk production and frequency of feeding. Another finding from a study found that breastfed infants may control the consumption of milk and learn to self-regulate their energy intake better than formula-fed infants (Plagemann, 2005).

2.8 Summary

Infant's health and nutrition has been a topic of extensive research. Many studies have been conducted to provide new knowledge and findings regarding this topic. Studies on breast milk composition have been conducted in many countries, but somehow limited in Malaysia. However, there is a few studies reported in Malaysia that aims to determine the composition of breast milk, but none has yet to determine breast milk carbohydrate composition across time point and within a feed (from fore- to hindmilk). As the composition of breast milk varies among mothers and population, it is beneficial to explore the trend of carbohydrate composition in breast milk among mothers living in Malaysia, and to seek information with infant's outcome.

Breast milk feedings are found to be associated with infant's growth and behaviour. Many studies have been conducted to see the association of breastfeeding with infant's growth and behaviour. Nevertheless, most of the studies do not consider which components of breast milk that are associated with growth and behaviour of infants. Besides that, there were no study conducted in Malaysia to determine the association of carbohydrate composition with growth and behaviour of infants. There is also lack of research that studying the trend changes on infant behaviour such as appetite in early human life.

Due to the gaps and limitations above, this study is conducted to determine the trend of changes of carbohydrate across different time-point and its association with growth and behaviour of infants. The findings of this study will contribute to knowledge and understanding of breast milk composition. Researcher will gain an insight how the changes of carbohydrate in breast milk across time point is associated with growth and appetite of infants. Moreover, the findings of this study will be beneficial to policy makers and manufacturers of formula milk. The composition of formula milk is created to be as close as possible to breast milk. However, a study found that 80% of formula products contain higher sugar amount (Palafox & Harris, 2017). The high amount of sugar will eventually increase adiposity level in later life (Khor et al., 2014). Based on standard for infant formula and formula (2015), there is no specific guidelines for lactose and oligosaccharide concentration in formula. The guideline is very general, which the formula milk should follow minimum and maximum level of carbohydrate. Due to this concern, knowledge and understanding on composition of breast milk, especially carbohydrate will be helpful to improve the composition of formula milk marketed in the industry.

CHAPTER 3
METHODOLOGY

3.1 Study Design

This was a longitudinal observational study, which data has been collected as part of a randomized controlled trial that (clinicaltrail.gov identifier: NCT01971216) investigating mother-infant signalling during breastfeeding (Shukri, Wells, Mukhtar, Lee, & Fewtrell, 2017). The present study aimed to determine the carbohydrate composition in breast milk in relation to infant's growth and behaviour.

3.2 Study Location

The target population sample of the study was Klang Valley. The breast milk samples and data on infant's factors were collected among first time mothers in the study location. All data including the biological samples (breast milk) were collected previously by researchers in MOMStudy. Klang Valley is an urban area located in the central region of Malaysia with a population of 1.7 million in 2019 (Department of Statistics Malaysia). The main ethnic groups in this area are Malay, Chinese and Indian which comprises of 45.0%, 43.2% and 10.3% respectively. According to Department of Statistics Malaysia 2019, the number of live births were recorded to be 6,575 births in the third quarter of 2019 in Klang Valley. The study location was chosen as the area recorded as highest birth rates and infant mortality and morbidity rates according to Malaysia Health Indicator (Malaysia Ministry of Health, 2014). Besides that, mothers in the area reported to have high initiation of breastfeeding (93.8%) and have fairly good knowledge on breastfeeding (Radzniwan et al., 2009).

3.3 Sample size

3.3.1 Sample size distribution

There was a total of 64 respondents. The human breast milk samples were collected at 3 different time-point which are week 2, week 4-6 and week 8-12. The breast milk samples were also collected prior and after feeding to collect foremilk and hindmilk samples. The milk samples from the first and end time point were chosen for this study. The available milk samples used was 90. The sample distribution number is shown in figure 2 and figure 3 below:

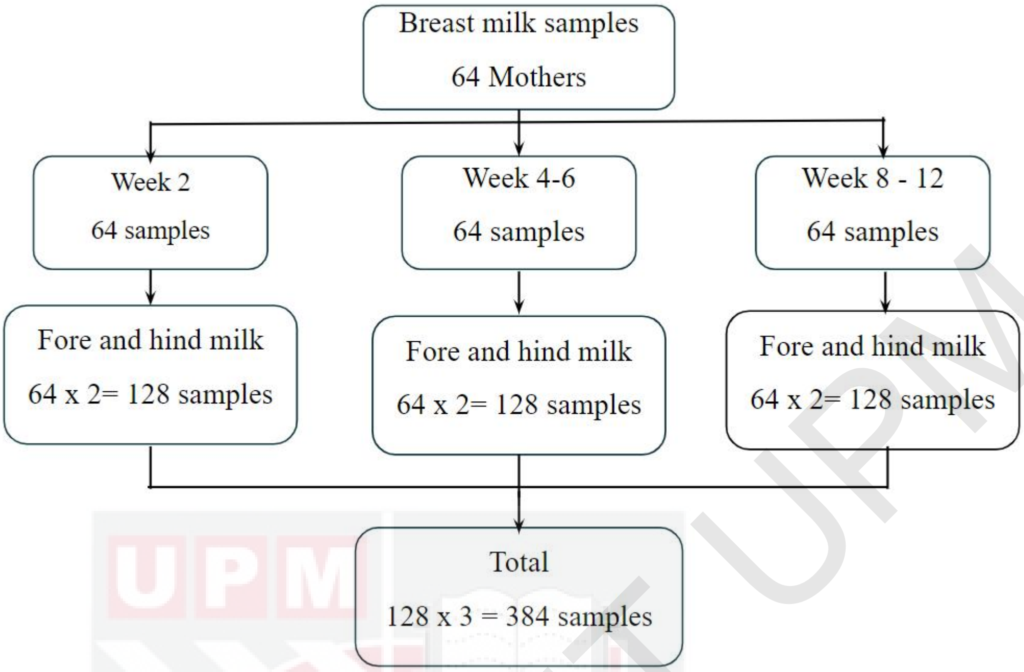

Figure 2: Breast milk samples for analysis of total carbohydrate

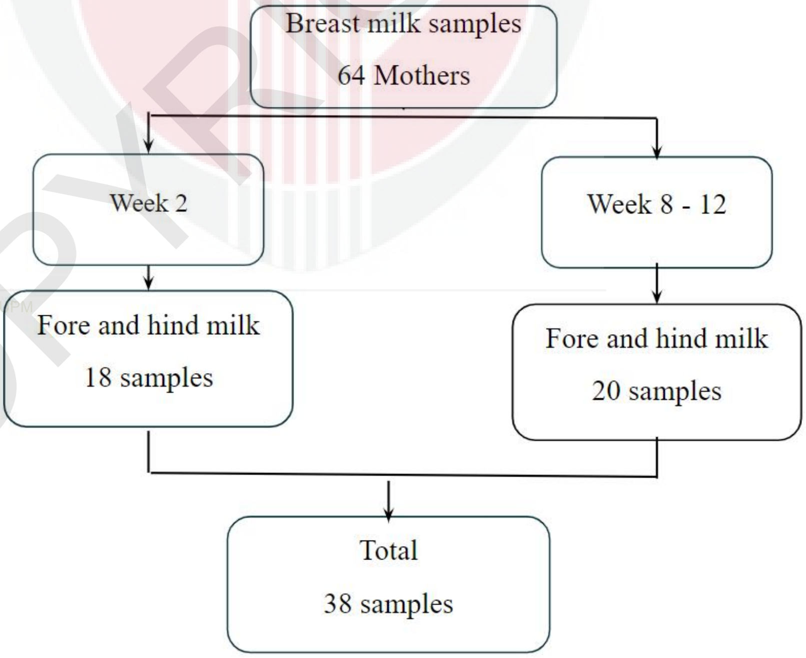

Figure 3: Breast milk samples for analysis of lactose

3.3.2 Correlation sample size calculation

Correlation sample size was calculated to determine the appropriate sample size required to study the association of carbohydrate composition with growth and behaviour of infants. The sample size was determined by using the following formula for hypothesis testing of two population proportions and correlation studies (Hulley et al., 2013).

$$\mathbf{N = [(Z\alpha + Z\beta)/\ C]^2 + 3}$$

Where

The standard normal deviate for $\alpha = Z\alpha = 1.96$

The standard normal deviate for $\beta = Z\beta = 1.28$

$C = 0.5 \times \text{In}[(1+r)/(1-r)]$

r = the expected correlation coefficient

Table 2: Correlation sample size calculation

Correlation studies	***r***	***p-value***	***N***
Correlation between total carbohydrate and BMI-for-age (BAZ) at week 4-6 (Mohd Shukri, N. H. et al. 2019)	0.306	0.019	108
Correlation between total carbohydrate and weight-for-age (WAZ) at week 8-12 (Mohd Shukri, N. H. et al. 2019)	0.257	0.048	155

Thus, the collection of breast milk from 64 mothers in MOMStudy is not sufficient to determine the association of carbohydrate composition with growth and behaviour of infants.

3.3.3 Comparison of sample size

The sample size collected was also compared with other studies on determination of carbohydrate composition across time-points to see if the collection of samples from 64 mothers sufficient to determine trend of changes of carbohydrate composition.

Table 3:Comparison of sample size

References	*N*
Variation in fat, lactose and protein in human milk over 24 hour and throughout the first year of lactation (Mitoulas et al., 2002)	17
Breast milk composition and infant nutrient intakes during first 12 months of life (Grote et al., 2016)	30
Carbohydrates in human milk and body composition of term infants during the first 12 months of lactation (Gridneva et al., 2019)	20

Therefore, the collection of breast milk from 64 mothers in MOMStudy is sufficient for determination of trend of changes of carbohydrate composition.

3.4 Respondents

Respondents of this study were mothers and infants who met the following inclusion and exclusion criteria based on MOMStudy protocol:

Table 4: The eligibity criteria for mothers and infants based on MOMStudy protocol

Mother	Infants
-Free of illness that can affect breastfeeding	-Full-term (37-42 weeks of gestation)
-Exclusively breastfeeding	-Birth weight of ≥ 2500 g -Exclusively breastfed -Free from illness that could affect nursing or growth

3.5 Data Collection

Data collection was completed between March 2014 and March 2015. Breast milk samples were collected during home visits which were conducted for 3 times which are in week 2, week 4-6 and week 8-12 during the postpartum period. During the visit, mothers were asked to express about 10-15 ml of breast milk before and after breastfeeding session to collect foremilk and hindmilk samples. Mothers were encouraged to message their breast prior to expressing milk to stimulate milk ejection. Milk samples were stored temporarily in milk storage containers. The containers were kept in an insulated box containing frozen silica pad during the visit. Then, the milk samples were transferred into 15 ml tubes and stored in freezer in the laboratory of the Faculty of Medicine and Health Sciences, Universiti Putra Malaysia at -80C degrees until analysis. Self-administered questionnaire and anthropometric measurement were also performed during the visit.

3.6 Analysis of samples

Total carbohydrate content was analysed using MIRIS Human Milk Analyzer method in MOMStudy Protocol whereas lactose component specifically will be analysed using Reversed Phase High Performance Liquid Chromatography (RP-HPLC) method in the current study (Sexton & Leann, 2004).

3.7 Preparation of sample

The stored samples were thawed at 22 °C for 30 minutes. The fat extraction was done by adding approximately 2 mL of diethyl ether. The solution was shaken while opening the valve periodically to allow the gas to escape. The aqueous and organic layers were allowed to separate. The bottom layer, the aqueous layer was collected. The extraction was repeated until no more fat was visible with the separation (Sexton & Leann, 2004).

3.8 Reagents and Chemicals

Reagents and chemicals were needed for preparation, analysis and quantification of lactose using reversed-phase HPLC. Diethyl ether was needed for extraction of fat from the breast milk samples in the preparation step. Besides that, DMSO (AR grade, Fisher) was needed for preparation of p-aminobenzoic solution with 4-aminobenzoic acid, Sigma with ≥99% purity which carry its function to attack reducing end of sugar that results in the ring opening. Furthermore, sodium cyanoborohydride, Sigma with 95% purity was also needed to break the double bond between carbon and nitrogen for the final product. The standards used was lactose with 0.01M. In the analysis, HPLC grade methanol was used as mobile phase. The adjustment of mobile phase pH was done by using glacial acetic acid of 99.8% purity (Sexton & Leann, 2004).

3.9 Determination of Total Carbohydrate

Total carbohydrate content comprises of both lactose and oligosaccharide. Total carbohydrate data was used as secondary data as the data was analysed for MOMStudy. The total carbohydrate content was analysed using the MIRIS Human Milk Analyzer (HMA) based on mid-infrared transmission spectroscopy. The calibration mode was set for homogenised human milk samples according to manufacturer's guidelines. The HMA provides data for total carbohydrate, including lactose and oligosaccharide of the breast milk (Casadio et al., 2010).

3.10 Determination of lactose concentration in breast milk

Lactose composition in breast milk was analysed using reversed phase high-performance liquid chromatography (HPLC) analysis. Procedure involved preparation of derivation sugar solution, specifically lactose and the experimental steps to prepare the samples for analysis using HPLC.

3.10.1 Preparation of derivation sugar

Derivation sugar solution from a mixture of sodium cyanoborohydride and p-aminobenzoic acid was used to derive the sugar molecules prior to analysis. The procedure on preparation of derivation sugar solution is shown in Figure 4. The procedure starts with heating the samples with reagents, and then cool to transfer it into 10ml volumetric flask, which eventually diluted with a mixture of methanol:water.

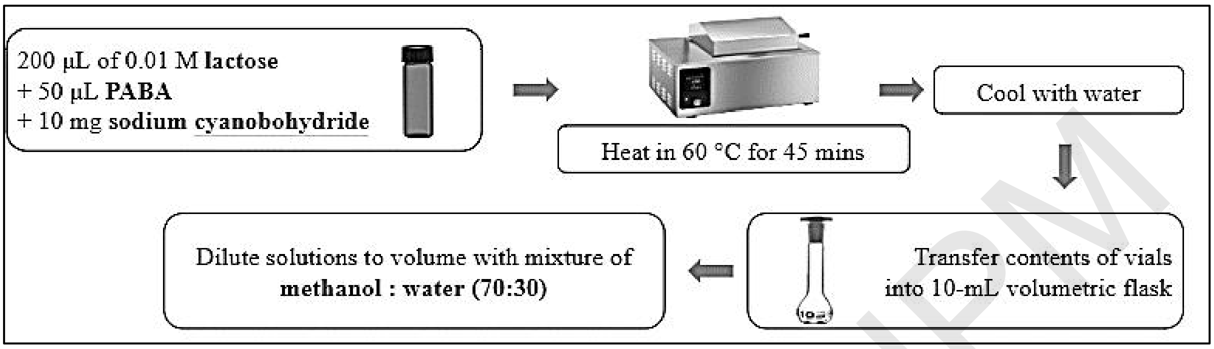

Figure 4: Preparation of derivation sugar

3.10.2 Experimental procedure

The breast milk samples were thawed in water bath prior to analysis. The samples were added with diethyl ether and shaken while opening the valve periodically to allow gas to escape for extraction of fat. The fat extraction procedure was repeated until no fat layer is visible. Derivation sugar solution and sodium cyanoborohydride were added into solution and 10 uL of solution was pipetted into HPLC vial. The retention time of the sample peak was compared to standard peak for determination of lactose. The regression analysis was performed to produce the equation of regression line and correlation coefficient. A standard curve was plotted for the quantification. From the standard curve, a formula was derived which is:

$$y=mx + c$$

y= area under graph
m= gradient
x= concentration of lactose
c= y-intercept

The concentration of lactose (x) can be determined with the known value of y, m and c. Area under the graph was used to measure the concentration (Sexton & Leann, 2004). Figure 5 shows an experimental procedure of the study. From the procedure, the samples diluted with mixture of methanol:water were injected into HPLC vials for HPLC analysis. The peak of sample was compared to standard peak for determination of lactose.

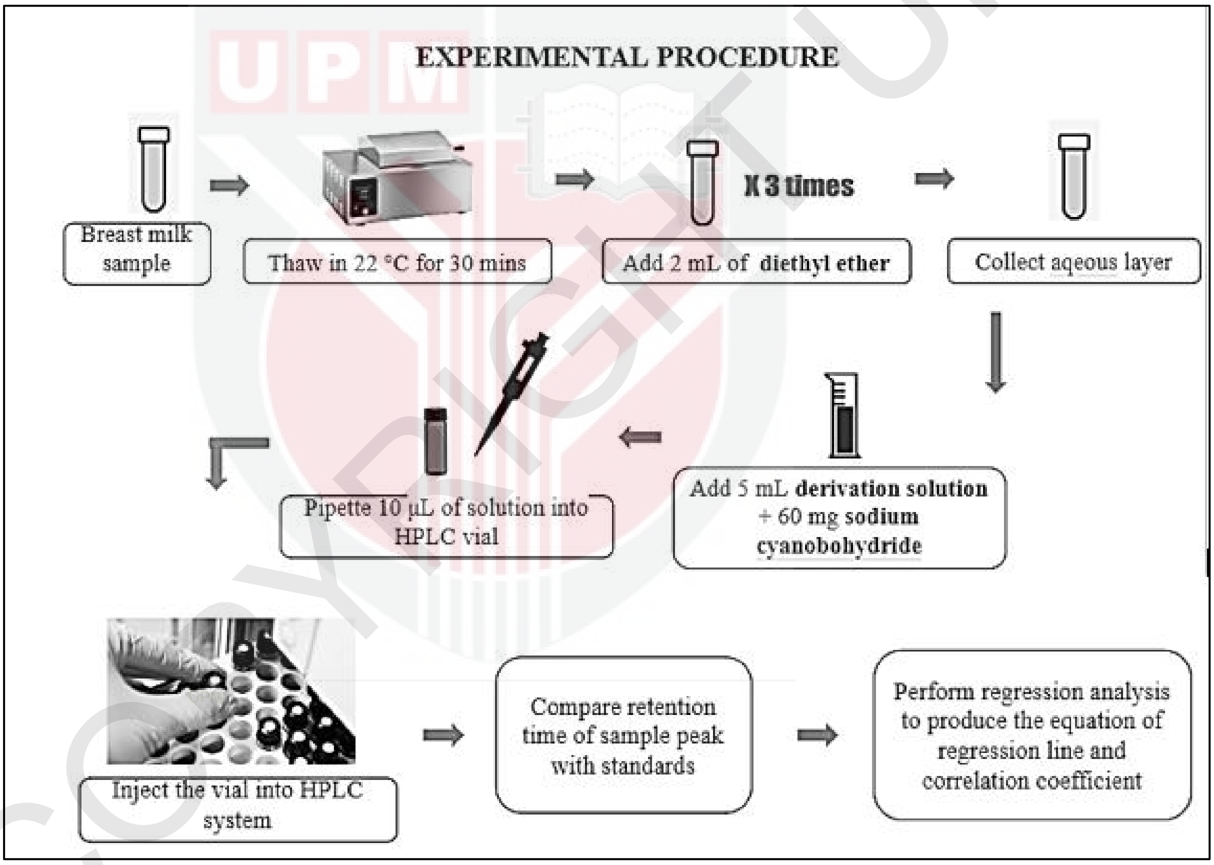

Figure 5: Experimental procedure

3.10.3 HPLC Condition

The basic HPLC system consist of a pump, column, detector and reading device. All HPLC separations was performed under isocratic conditions with a flow-rate of 0.5 mL/min. The mobile phase was composed of 85:15 methanol: water mixture. The pH of the mobile phase was adjusted to 5.5 with glacial acetic acid. Each component of the mobile phase was thoroughly degassed for approximately 30 minutes with helium gas. The C8 column was used for detection of sugar molecules. The diode array detector was used with a wavelength range that set to 300-360 nm.

3.11 Measures

Self-administered questionnaire was used to determine the growth and behaviour of infants. The data was used to seek the association with carbohydrate composition in breast milk. The data was collected in MOMStudy.

3.11.1 Infant's growth

Infant's body weight and height were measured to determine weight-for-age, BMI-for-age and weight gain of infants. The infant's body weight was measured using digital infant weighing scale (Seca 834, Germany), which was calibrated regularly throughout the data collection period. A towel was placed over the scale before the scale was resetting to zero. All infants were weighed nakedly.

Infant's recumbent length was measured using Infant Length Measuring Mat (Rollameter 60, UK) which has one fixed hardboard on one end and movable measuring tape on vertical plane on the other end. The infants were measured in Frankfurt Plane position, which the infant's lay supine with the head against the fixed hardboard and the body parallel to board's axis. The infant's legs were straightened by holding the legs at the ankle with one hand while the other hand apply downward gentle pressure over the legs. Mothers were asked to check for the position to make sure the head was still in position and touching the head plane. The measurement was taken once the infant was in straight line position with feet straights where the vertical plane was mounted to touch the soles of infant's feet with toes pointing directly upward. The measurement was read from the red arrow in the reader window.

Both instruments used are reliable and valid to measure infant's length and weight (Rajaah et al., 2010).

All anthropometric measurements were taken three times and the mean value was used in the analysis. Measurements were taken at 3 different time points which are week 2, week 6-8 and week 12-14. Table 5 shows the classification of anthropometric indicators based on WHO (2007).

Table 5: Classification of growth chart

Anthropometric Indicators			
Z score	**Weight-for-age**	**Height-for-age**	**BMI-for-age**
<-3SD	Severely underweight	Severely stunted	Severely wasted
≥-3SD to <-2SD	Moderately underweight	Stunted	Thinness
≥-2SD to ≤-1SD	Mildly underweight	Normal	Normal
≥-1SD to ≤2SD	Normal		Overweight
>2SD to ≤3SD			Obese
>3SD			Severely obese

Source: WHO Growth Standard (2006)

3.11.2 Infant's behaviour

3-day Infant Behaviour Diary was used to measure infant behaviour. There is a total of 72 hours record, which divided into 15 mins segments. Mothers were asked to shade according to the infant's behaviour which include sleeping, crying, fussy awake, content and feeding. Each behaviour has different shading pattern. Description on how to fill was explained to mothers by researcher. Written description was also included in the questionnaire (Barr et al., 1988). Data on frequency and duration of feeding and duration of sleeping were obtained from this diary.

Baby Eating Behaviour Questionnaire (BEBQ) is a modified version of Child Eating Behaviour Questionnaire (CEBQ) that is used to measure appetite of infants.

It consists of 18 items which measures 4 appetite traits, which are enjoyment of food (4 items), food responsiveness (5 items), slowness in eating (4 items) and satiety responsiveness (5 items). The mothers were asked to rate based on five point-Likert scale which range from 1 (never) to 5 (always). The appetite traits are defined as i) Enjoyment of Food (EF): the infant's liking for milk or how much the infant enjoys the feeding time in general, ii) Food Responsiveness (FR): infant responsiveness to maternal cues for feeding, as well as his/her demandingness for feeding, iii) Slowness in Eating (SE): the pace of feeding or how slowly/quickly an infant feeds; and Satiety Responsiveness (SR): a measure of the extent to which the infant gets full easily. From the appetite traits, mean score will be calculated for each subscale. The higher mean score indicates greater expression of the traits. BEBQ is a useful and comprehensive tool used to measure appetite of infants (Llewellyn et al., 2011).

3.12 Statistical analysis

The data was analysed using IBM SPSS Statistics Software version 20. The descriptive data were presented for all variables including independent and dependent variables. The value of mean and standard deviation was reported. Independent sample t-test was used to compare the composition of breastmilk within feeding (foremilk vs hindmilk), while ANOVA test was used to compare the concentration at 3 different time points. The inferential statistical test was performed to measure the association of independent and dependent variables. Normality test was conducted to see if the data was normally distributed. Pearson products moment correlation was used to see the association. The level of statistical significance was set at $p<0.05$.

CHAPTER 4

RESULTS AND DISCUSSION

This chapter provide descriptive and inferential analysis of the study variables. The aim of this chapter is i) to present descriptive data on carbohydrate composition, infant's growth and behaviour and ii) to discuss the association of carbohydrate composition with infant's growth and behaviour. The description begins with descriptive characteristics of study population, followed by data on infant's growth and infant's behaviour. Then, the trend of changes of total carbohydrate and lactose across different time-point as well as within feeding are further described in this chapter. Next, the associations of carbohydrate composition with infant's growth and behaviours were presented as bivariate analysis and discussed. The number of subjects participated in this study was 64.

4.1 Sociodemographic Characteristic

4.1.1 Demographic Background

The demographic characteristics are described in Table 6. Out of 64 subjects, 60.9% subjects were female and 39.1% were male. Majority of the subjects were Malay (93.8%), followed by Chinese (3.1%), Indian (1.6%) and Bumiputera (1.6%).

Table 6: Demographic background of subjects

Characteristics	n (%)
Sex of subjects	
Male	25 (39.1)
Female	39 (60.9)
Ethnicity	
Malay	60 (93.8)
Chinese	2 (3.1)
Indian	1 (1.6)
Bumiputera	1 (1.6)

4.1.2 Socioeconomic Background of Subject's Mothers

Table 7 shows the sociodemographic background of mothers. The mean age of mothers was 26.6 ± 2.79 years old. The highest education level is postgraduate whereas the lowest is secondary or primary school. Most of mothers were bachelor degree graduates (.9%) and the rest were secondary school graduates (14.1%), pre-university of diploma graduates (12.5%), postgraduates (10.9%) whereas the least was primary school graduate (1.6%). Nearly half of the subject's mothers had household income of >RM 8301 (45.3%) whereas minority of mothers had household income of <RM 3800. For the occupation, majority of the mother work in private sector (37.5%).

Table 7: Socioeconomic background of subject's mothers

Demographic background	n (%)	Mean±(SD)
Mother's age		26.66±2.79
20-25	21 (32.8)	
26-30	38 (59.4)	
31-35	5 (7.8)	
Mother's educational level		
Primary school	1 (1.6)	
Secondary school	9 (14.1)	
Pre-University/Diploma	8 (12.5)	
Bachelor degree	39 (60.9)	
Postgraduate	7 (10.9)	
Mother's occupation		
Public sector	18 (28.1)	
Private sector	24 (37.5)	
Self-employed	1 (1.6)	
Housewife	7 (10.9)	
	14 (21.9)	
Household income		
<RM 3800	13(20.3)	
RM 3801 – RM 8300	22(34.4)	
>RM 8301	29(45.3)	

4.2 Infant's growth

The infant's growth at birth and at week 8-12 are reported in the table below. From the data, the proportion of normal weight-for-age was 96.9% whereas 3.2% of subjects were classified as underweight. The finding of this study was compared to national study data. Based on NHMS (2016), 84.7% of children were in the normal range for weight-for-age, whereas 13.7% of children were underweight.

For the height-for-age (HAZ) data, all subjects participated in this study had normal length-for-age. This shows that exclusively breastfed infants had a positive outcome on infant's length. As compared to NHMS data (2016), the prevalence of infants with normal HAZ was 76.0%. Besides that, 89.1% of subjects had normal

BMI-for-age, whereas 7.9 % were wasted and 3.2% were severely wasted. This data is quite similar to NHMS data which reported that the prevalence of wasting was 11.2%.

From the data, underweight and wasting was more prevalent compared to overweight or obese. However, the data was comparatively lower compared to NHMS 2016.

Table 8: Infant's growth at week 8-12

Infant's growth at week 8-12	n (%)	Mean ± SD
Weight-for-age		-0.50 ± 0.87
Overweight and obese	0 (0.0)	
Normal	62 (96.9)	
Underweight	2 (3.2)	
Severely underweight	0 (0.0)	
Height-for-age		0.40 ± 1.02
Tall	0 (0.0)	
Normal	64 (100.0)	
Stunted	0 (0.0)	
Severely stunted	0 (0.0)	
BMI-for-age		-0.99 ± 1.01
Obese	0 (0.0)	
Overweight	0 (0.0)	
Possible risk for overweight	0 (0.0)	
Normal	57 (89.1)	
Wasted	5 (7.9)	
Severely wasted	2 (3.2)	

4.3 Infant's behaviour

4.3.1 Appetite

The appetite traits of infants are tabulated in the table 9 below. The traits of enjoyment of food (EF) and food responsiveness (FR) indicate greater appetite, whereas the traits of slowness in eating (SE) and satiety responsiveness (SR) indicate appetite control. Based on the data, EF and FR showed high mean score of 4.32 ± 0.40 and 3.15 ± 0.72 respectively compared to SE (2.99 ± 0.73) and SR (2.51 ± 0.54). Thus, infants expressed greater appetite traits compared to appetite control traits. The mean score for general appetite was 4.17 ± 0.83.

Table 9: Infant's appetite traits at week 2

Appetite traits	Mean ± SD
Enjoyment of food	4.32 ± 0.40
Food responsiveness	3.15 ± 0.72
Slowness in eating	2.99 ± 0.73
Satiety responsiveness	2.51 ± 0.54
General appetite	4.17 ± 0.83

4.3.2 Feeding and sleeping behaviour

Data on infant’s behaviours was collected using 3-Day Infant’s Diary. The mean feeding duration at week 2 was 246.68 ± 85.93 min/day. The mean duration was compared to another study, which the study shows that feeding duration at the first 2 weeks of life was 162 ± 50 min/day (de Carvalho et al., 1982).

The mean feeding frequency of infants was 10.32 ± 2.94 times/day. This data is consistent with other studies on feeding frequency. Khan et al., (2013) reported that the frequency of breastfeeding ranged from 11 to 19 times among infants age 1-6 months. Another study reported that the mean frequency of feeding was 11 ± 3 times/day, which ranged from 6 to 18 times for infants aged 2 to 5 months (Kent et al., 2006).

The mean sleeping duration of infants at week 2 was 830.38 ± 127.90 min/day. The sleeping duration was compared to another study from Singapore which found that the mean duration of sleeping was 724.8 ± 3.91 hours/day (Zhou et al., 2015).

Table 10: Infant's feeding and sleeping behaviour at week 2

Infant's behaviour	Mean ± SD
Feeding duration (min/day)	246.68 ± 85.93
Feeding frequency (times/day)	10.32 ± 2.94
Sleeping duration (min/day)	830.38 ± 127.90

There was high variability in the data as the SD value was high. It is possibly due to low response rate among mothers. Some mothers did not complete the 3-Day infant's diary. This may be regarded as limitation of self-administered questionnaire, which researcher has limited control over the response rate.

4.4 Total carbohydrate

The mean total carbohydrate data is presented in Table 11. From the data, the range of mean total carbohydrate concentration across time-point was 7.00 – 7.10 g/dL. This data is consistent with the finding from a recent study which reported that the mean value of carbohydrate concentration was 7.09 ± 0.43 g/dL (Czosnykowska-łukacka et al., 2018). In contrast, the mean total carbohydrate reported in another study was 8.67 ± 9.2 g/dL at 2 months (Gridneva et al., 2017). However, the difference in the concentration might be due to different method used to analyse the samples as the study used UV spectrophotometric whereas this study used MIRIS human milk analyser.

The trend of carbohydrate composition across time-point was also determined in this study. There was no significant difference between total carbohydrate concentration across time-points ($p > 0.05$). This finding is supported by a recent study which found that total carbohydrate concentration was stable in 1-12 months of lactation (Czosnykowska-łukacka et al., 2018).

Table 11: Trend of changes of total carbohydrate across time-points

Infant's age	**Foremilk (g/dL) (Mean ± SD)**	**Hindmilk (g/dL) (Mean ± SD)**	**F**	***p-value***
Week 2	7.05 ± 0.42	7.00 ± 0.44	1.126	0.239
Week 4-6	7.03 ± 0.45	7.02 ± 0.46	2.134	0.150
Week 8-12	7.04 ± 0.52	7.10 ± 0.72	0.458	0.501

Furthermore, the total carbohydrate concentration was also compared between foremilk and hindmilk. The results are tabulated in table 12. There was no significant difference of total carbohydrate between foremilk and hindmilk ($p>0.05$).

Table 12: Trend of changes of total carbohydrate between time-point

Total Carbohydrate (g/dL)	**Mean ± SD**	**t**	***p-value***
Week 2			
Foremilk	7.05 ± 0.42	-0.870	0.388
Hindmilk	7.00 ± 0.44		
Week 4-6			
Foremilk	7.03 ± 0.45	-0.219	0.828
Hindmilk	7.02 ± 0.46		
Week 8-12	7.04 ± 0.52		
Foremilk	7.10 ± 0.72	0.749	0.457
Hindmilk			

4.5 Lactose

Lactose was determined by using lactose standard curve with concentration ranging from 0.00036 – 0.01134 mg/ml. From the concentration, ta linear calibration curve was plotted with the equation of y = 6212.6x + 13.156 (R^2= 0.9741) as shown in Appendix 1. The lactose concentration in the samples were expressed as g/dL.

The mean lactose concentration was presented in table 13. The lactose concentration was compared with other studies. A recent cohort study that followed up mothers for first 12 months of lactation found that average concentration of lactose in breast milk was 6.8 g/dL, which ranged from 5.9 to 7.6 g/dL 9 (Gridneva et al., 2019). Another study also reported that the mean lactose concentration in mature milk was 7.1 g/d (Chang et al., 2015). This shows that the lactose concentration found in this study is consistent with other studies.

Table 13: Concentration of lactose

Infant's age	Foremilk (g/dL) (Mean ± SD)	Hindmilk (g/dL) (Mean ± SD)
Week 2	6.72 ± 0.57	6.77 ± 0.32
Week 8-12	6.81 ± 0.32	6.44 ± 0.92

In order to study the trend of changes of lactose across different time-point, the lactose concentration was compared between week 2 and week 8-12. The data was presented in table 14. There was no significant difference between lactose concentration across different time point (p= 0.267). This result is consistent with previous studies which compare the lactose concentration throughout 12 months of lactation. A cohort study on composition in breast milk in the first 12 months of lactation found that the concentration did not differ between 2 and 12 months (Gridneva et al., 2019). Another study of breastmilk concentration across different time-points in China also reported and the concentrations are consistent throughout time (Yang et al., 2014). This shows that lactose concentration is consistent across time-point up to 12 months. This statement is support by a review on breast feeding which stated that lactose content is constant in mature milk, which is after 21 days of postpartum period (C. R. Martin et al., 2016).

Table 14: Trend of changes of lactose across time-points

Infant's age	Lactose (g/dL) Mean ± SD	t	*p-value*
Week 2	7.23 ± 0.14	2.24	0.267
Week 8-12	7.07 ± 0.25		

The lactose concentration was also compared between foremilk and hindmilk to study the trend of changes within feeding in table 15. No significant difference was found for lactose concentration between foremilk and hindmilk (p=0.833) and (p=0.547). Lactose concentration shows no significant changes between foremilk and hindmilk (Saarela et al., 2005). Another recent study also found that there was no difference in lactose concentration for pre and post feeds (Gridneva et al., 2019).

Table 15: Trend of changes of lactose within feeding

Lactose (g/dL)	Mean ± SD	t	*p-value*
Week 2			
Foremilk	6.68 ± 0.91	0.230	0.833
Hindmilk	6.77 ± 0.49		
Week 8-12			
Foremilk	6.82 ± 0.41	0.645	0.547
Hindmilk	6.48 ± 1.08		

From the finding, lactose concentration is stable across time-point and within feeding. The stable concentration of lactose is important to maintain constant osmotic pressure in breast milk (C. R. Martin et al., 2016). The synthesis of lactose will create osmotic drag which facilitate milk production and secretion, thus lactose is essential for milk production as it drives osmotic force in the formation of milk volume (Lo et al., 2003).

However, there were several limitations occur during the analysis. Lactose concentration was not in the range of standard curve due to lack of time to repeat the tests with various concentration as the results produced from each analysis were not consistent. Optimization is needed for the results to be consistent to produce appropriate standard curve. The optimization can be made by altering the concentration of p-aminobenzoic acid solution, the volume of mobile phase used to dilute samples before injection or can be made by altering the hydrolysis duration and temperature.

4.6 Association of carbohydrate composition with growth

Table 16 presents the association of carbohydrate composition with growth of infants. From the table, a positive correlation was found between total carbohydrate and weight of infants (r=0.314, *p*=0.015). However, no significant association was found between lactose and weight of infants (p>0.05). Besides, carbohydrate composition was not significantly associated with other growth parameters, namely length and BMI of infants.

A large cohort study by Prentice et al., (2016) found that total carbohydrate was positively associated with weight and BMI, but not length of infants. The significant association of total carbohydrate and BMI might be due to high proportion of ingested carbohydrate that promotes glycogen and fat storage and it is also possible that infant feel less satiated and drink large volume of milk that results in weight gain. This study speculated that other confounders could explain the weight gain or adiposity as no apparent association was found with infant's length even though weight gain and stature growth were closely linked to infancy. The possible confounders could be maternal characteristics. In the other hand, lactose was positively correlated with infant weight, BMI and adiposity gains. The growth regulating effects of lactose was credited to the effect of prebiotic on gut microbiome which results in growth of lactobacilli and bifidobacterial (Pierce et al.,, 2004). These activities may improve minerals absorption and promote intestinal health.

Another cohort study was also conducted in Australia to study the association of carbohydrate composition in breast milk with infant's growth (Gridneva et al., 2019). This study reported that total carbohydrate was associated with greater infant's length and weight, whereas lactose was not significantly associated with body composition of infants. This finding is supported by several studies [(Goran et al., 2017) & (Mitoulas et al., 2002)]. The lack of association could be due to the primary role of lactose in maintaining osmotic pressure in breast milk (C. R. Martin et al., 2016).

Table 16: Pearson correlation of carbohydrate composition with growth of infants at week 8-12

Growth of infants	**Total carbohydrate (g/dL)**		**Lactose (g/dL)**	
	r	***p- value***	***r***	***p- value***
Weight (kg)	0.314	0.015	-0.768	0.075
Length (cm)	0.233	0.073	-0.641	0.170
BMI	0.218	0.094	-0.671	0.145

4.7 Association of carbohydrate composition with behaviour

4.7.1 Appetite

Table 17 show the association of carbohydrate composition with infant's growth and behaviour. Based on the results, there was no association found between total carbohydrate and lactose with appetite traits. However, there was no study reported on the association of carbohydrate composition with appetite traits of infants. This study hypothesised that carbohydrate is associated with appetite as previous study explained that appetite and hunger may be direct functions of dietary carbohydrate due to its effect in insulin induced hypoglycaemia (Geiselman & Novin, 1982). Moreover, another study conducted among breast-fed baby found that high lactose feeds are associated with rapid gastric emptying that lead to appetite and hunger (Woolridge & Fisher, 1988). However, the role of carbohydrate in breast milk on appetite is yet to be discovered. From the data, the greater appetite traits may be contributed by other components in breast milk such as other macronutrients or hormone as a recent study found that adiponectin, whey protein and casein were associated with gastric emptying that lead to hunger and appetite (Gridneva et al., 2017).

Table 17: Pearson correlation of carbohydrate composition with appetite traits of infants at week 2

Appetite traits	**Total carbohydrate (g/dL)**		**Lactose (g/dL)**	
	r	***p- value***	***r***	***p- value***
Enjoyment of food	-0.164	0.210	0.153	0.847
Food responsiveness	-0.248	0.056	-0.442	0.558
Slowness in eating	-0.253	0.051	-0.359	0.641
Satiety responsiveness	-0.119	0.365	-0.556	0.444
General appetite	-0.053	0.688	-0.344	0.656

4.7.2 Feeding and sleeping behaviour

There was no significant association found between carbohydrate composition with feeding duration of infants. This finding is supported by previous study which found that average lactose concentration was not associated with duration of feeding (p=0.79), possibly due to its role in maintaining osmotic pressure of breast milk (Khan et al., 2013).

No significant association was also found between carbohydrate composition with feeding frequency. This finding is supported by a previous study which found that lactose concentration in breast milk was not associated with frequency of feeding (p= 0.97). However, a significant association between lactose in breast milk with feeding frequency was found in other studies [(Khan et al., 2013) & (Gridneva et al.,2019)]. Infants who were frequently breastfed had high intake of lactose. It is because high lactose feeds was associated with gastric emptying rate, which regulate appetite in infants [(Welsh & Hall, 1977) & (Woolridge & Fisher, 1988)].

Furthermore, no significant association was found between carbohydrate composition with sleeping duration. However, there was no study conducted to determine this association. This study hypothesised that carbohydrate is associated with sleeping duration of infants as previous study reported that high carbohydrate intake was associated with reduced sleep onset latency, slow wave sleep and increased rapid eye movement, which occur with greater sleeping duration at night (St-Onge, Mikic, & Pietrolungo, 2016).

Table 18: Pearson correlation of carbohydrate composition with feeding and sleeping behaviour of infants at week 2

Infant's behaviour	**Total carbohydrate (g/dL)**		**Lactose (g/dL)**	
	r	***p- value***	***r***	***p- value***
Feeding duration (min/day)	0.128	0.413	-0.765	0.235
Feeding frequency (times/day)	-0.226	0.213	-0.324	0.676
Sleeping duration (min/day)	0.113	0.469	0.748	0.252

In summary, the average total carbohydrate and lactose concentration were within the range compared to previous studies. The concentration was stable across time-point and within a feeding, which is important to maintain the osmotic pressure in breast milk. Besides, around 90% of infants had normal WAZ, HAZ and BAZ which could be hypothesized that exclusive breastfeeding is associated with optimal growth of infants. Infants in this study also portrayed greater appetite traits compared to appetite control traits. From the analysis, a significant positive relationship was found between total carbohydrate and weight of infant whereas no significant association was found between total carbohydrate and other growth parameters, namely length and BMI of infants. Apart from that, lactose was not significantly associated with weight, length and BMI of infants. Lastly, no significant correlation was found between carbohydrate composition and behaviour of infants. The insignificant results could be due to small sample size, which do not have enough power to show the association.

CHAPTER 5

CONCLUSION

Based on the findings, the carbohydrate composition of breastmilk is consistent throughout time-points and within a feeding. Besides, exclusively breastfed infants in this study shows positive outcomes in growth especially for HAZ. This shows that breastfeeding plays an important role in the normal growth of infants. The findings of this study may be beneficial for the formula milk industry to maintain the total carbohydrate and lactose concentration for the consumption of infants that have limited or no access to human breast milk due to certain circumstances. Total carbohydrate was found to be positively correlated with infant's weight as lactose feeds promote glycogen and fat storage. No significant association was found between carbohydrate composition with other growth parameter as well as the behaviour of infants.

5.1 Limitations

There were several limitations identified in this study. Firstly, the research dealing with the small sample size. This small sample size has limited the ability to study the association of carbohydrate composition with infant's growth and behaviour. The association was not found in this study as it is supported by the findings of a larger study (Butte et al., 1984).

Furthermore, the concentration of total carbohydrate and lactose were measured at different time-point, which the determination of total carbohydrate was measured in 2016 whereas lactose determination was measured in this study. Therefore, there might be interference with lactose concentration in breast milk during long-time storing in freezer.

Besides, this study did not take into account the milk intake or volume of feeds of infants to measure the actual intake of milk that may be associated with growth and feeding behaviour of infants.

Other than that, the research has a limited time to perform the analysis as there was a need to dealings with environmental current situation which is Pandemic Covid-19. The research time frame needs to be adjusted and shorten as Malaysian government implement Movement Control Order (MCO) which restricted a lot of activity including research activity. Other than that, post MCO, Universiti Putra Malaysia (UPM) also implement a restrict procedure in align with Health regulatory advice. So, it compressed the time frame to be only about 1 and half month. Therefore, unfortunately, samples cannot be analysed with a proper time frame and optimization of lactose in breast milk were not able to be performed.

5.2 Recommendations

Future research should incorporate large and appropriate number of sample size of study population to determine the association. Besides, the allocation of enough time is required to perform analysis of all samples. Enough allocation of time is also needed for the optimization of lactose using HPLC to develop the most suitable and reliable procedure for the analysis. Moreover, future research should also include other potential confounding factors that may contribute to the growth and behaviour of infants such as milk intake or volume of feeds of infants. Furthermore, it is recommended for future study to analyse other carbohydrate composition such as oligosaccharide to study its association with infant's growth and behaviour. Last but not least, analysing total carbohydrate, lactose and oligosaccharide during similar timeframe is highly recommended for future studies.

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APPENDIX 1

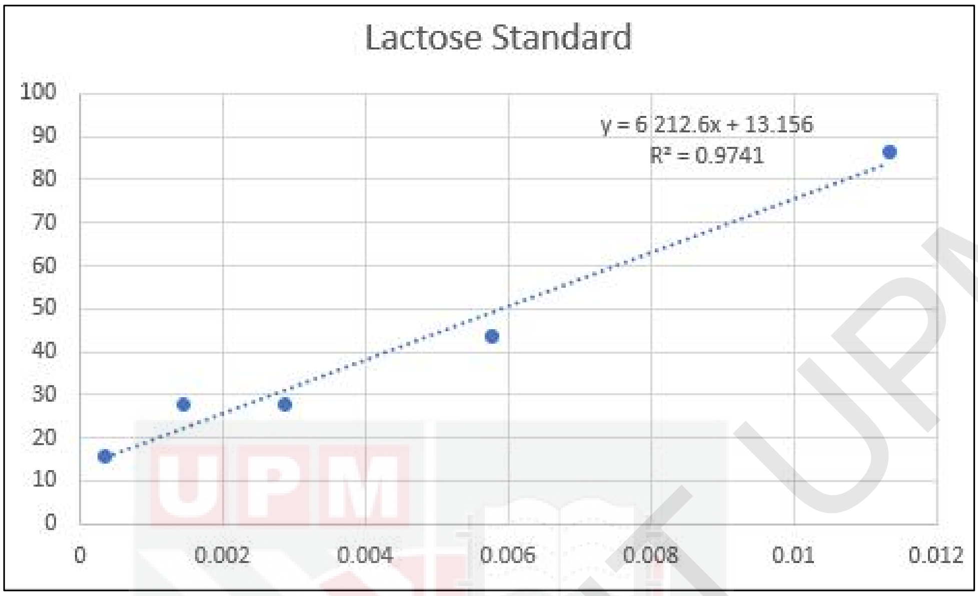

Figure 6: Standard curve of lactose