Mother-infant signalling during lactation following late preterm and early term delivery: An investigation of infant feeding from physiological, psychological, anthropological and microbiological perspectives

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Declaration

I, Jinyue Yu, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Signature: 

Date: 2022/4/6

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Abstract

Breastfeeding is a dynamic process involving signalling between mother and offspring through biological (breast milk) and non-biological (behavioural) pathways. Maternal stress is one modifiable variable that could influence signalling and negatively affect breastfeeding and infant growth. Understanding this dynamic process may facilitate targeted interventions that promote breastfeeding and improve infant and maternal outcomes.

My research investigated mother-infant signalling following late preterm (LP) and early term (ET) delivery – a situation associated with higher maternal stress and greater breastfeeding difficulty than full-term delivery - with interpretation from physiological, psychological, and anthropological perspectives. The acute effects of five relaxation techniques on physical and perceived relaxation among 20 Chinese primiparous breastfeeding mothers were compared in a pilot study. A relaxation meditation recording was the most effective intervention, and was subsequently tested (versus no intervention) in a randomised controlled trial in 96 healthy primiparous mother-infant pairs following LP or ET delivery. Relaxation therapy significantly reduced maternal stress and increased infant weight gain from 1-8 weeks. There was a significant interaction with greater intervention effects on weight gain in girls. Mothers of girls used the intervention more frequently with a trend for higher milk fat and energy at 8-weeks. Infant length gain and milk energy and fat at 8-weeks were also non-significantly higher in the intervention group.

Additionally, my research innovatively explored the role of the microbiome in mother-infant signalling. The relaxation intervention led to reduced maternal gut and breast milk microbiome diversity whilst increasing infant gut microbiome diversity; it also influenced the change in microbiome from 1-8 weeks.

The study findings have practical implications for supporting breastfeeding mothers following LP and ET delivery. The relaxation meditation tape is a simple, practical tool that could easily be used in clinical settings. The research findings also contribute to understanding the mechanisms of mother-infant signalling during early life.

Impact Statement

Breastfeeding is one of the most effective ways to ensure infant health and survival. The World Health Organization (WHO) recommends exclusive breastfeeding (EBF) for the first six months of life. Although there is much research interest in the effectiveness of interventions on promoting breastfeeding during early infancy, the global EBF rate remains below targets. As reported by WHO, nearly two out of three infants are not exclusively breastfed for the recommended 6 months and this rate has not improved in two decades.

Mother-infant signalling during lactation is one of the prominent mother-infant factors that may influence breastfeeding outcomes. Understanding this signalling can be particularly important for late preterm and early term infants and their mothers, given the greater morbidity, higher rates of breastfeeding complications, and higher maternal stress seen in this population, which is expected to lead to greater mother-infant 'tension' over resources. In this thesis, I investigated mother-infant signalling during breastfeeding by manipulating maternal psychological state using a relaxation intervention. Results of my research confirmed the effects of the relaxation meditation tape on reducing maternal stress and improving infant weight gain from 1-week to 8-weeks postpartum. Moreover, the intervention can result in altered alpha diversity of microbiome in maternal breast milk, gut, and infant's gut.

The implications of my research include four parts. First, a simple and non-invasive relaxation therapy given to breastfeeding women showed beneficial effects on reducing

maternal stress and increasing infant weight gain and could be used in mothers of late preterm and early term infants both in hospital and after discharge. Second, my research in this vulnerable and understudied group of infants suggests more clinical attention and support needs to be provided in order to improve their breastfeeding outcomes. Third, whilst the results of my research highlighted the value of considering anthropological theories in biomedical research, anthropological research may also benefit from considering biomedical research and using experimental study designs, considering most anthropological research is observational or involves animals. Finally, my research provides a better understanding of the complex interplay of psychological, behavioural, and anthropological factors involved in mother-infant signalling which influence breastfeeding outcomes. In particular, my research innovatively investigated the microbiome as a potential signal between mother and infant using an experimental approach with preliminary results suggesting a plausible role which justifies further investigation.

Publications and Presentations

Publications from the PhD project

1. **Jinyue Yu**, Jonathan Wells, Zhuang Wei, Mary Fewtrell, 2019. Effects of relaxation therapy on maternal psychological state, infant growth and gut microbiome: protocol for a randomised controlled trial investigating mother-infant signalling during lactation following late preterm and early term delivery. *International Breastfeeding Journal,* 14(1), 1-9
2. **Jinyue Yu**, Jonathan Wells, Zhuang Wei, Mary Fewtrell, 2019. Randomized trial comparing the physiological and psychological effects of different relaxation interventions in Chinese women breastfeeding their healthy term infant. *Breastfeeding Medicine, 14(1),* 33-38.

Publications from the period as visiting scholar at Zhejiang University

3. **Jinyue Yu**, Qian Yi, Leying Hou, et.al. 2021. Transition of Lipid Accumulation Product Status and the Risk of Type 2 Diabetes Mellitus in Middle-Aged and Older Chinese: a National Cohort Study. *Frontiers in Endocrinology.* 12.
4. **Jinyue Yu**, Qian Yi, Ge Chen, et.al.2021. The Visceral Adiposity Index and risk of type 2 diabetes mellitus in China: A national cohort analysis. *Diabetes/Metabolism Research and Reviews.*:e3507.
5. Xinxin, Ye, Wan Ye, **Jinyue Yu**, Yuzhen Gao, Ziyang Ren, Lanzhen Chen, Ao Dong et al. The landscape of COVID-19 vaccination among healthcare workers at the first round of COVID-19 vaccination in China: willingness, acceptance and self-reported adverse effects. Human Vaccines & Immunotherapeutics (2021): 1-11.

Publications during the COVID-19 pandemic in China

6. **Jinyue Yu**, Mingyue Gao, Zhuang, Wei, Jonathan Wells, and Mary Fewtrell. 2022. The impact of the Covid-19 on maternal delivery experiences and breastfeeding practices in China: data from a cross-sectional study. BMC Pediatrics, 22(1):1-1.
7. Zhuang, Wei, Mingyue Gao, Mary Fewtrell, Jonathan Wells, and **Jinyue Yu**, 2021.Maternal mental health and well-being during the COVID-19 pandemic in Beijing, China. World Journal of Pediatrics 17, no. 3 (2021): 280-289.
8. **Jinyue Yu**, Zhuang Wei, Olga Lukoyanova, Tatyana Borovik, and Mary S. Fewtrell, 2020. Maternal Infant-Feeding Attitudes, Infant Eating Behaviors, and Maternal Feeding Choice at 3 and 6 Months Postpartum: A Comparative Multicenter International Study. *Breastfeeding Medicine*. 15(8), 528-534.

Systematic reviews published during the PhD study period

9. **Jinyue Yu**, Peige Song, Yan Zhang, and Zhuang Wei, 2020. Effects of Mindfulness-Based Intervention on the Treatment of Problematic Eating Behaviors: A Systematic Review. *The Journal of Alternative and Complementary Medicine*, 26(8), 666-679.
10. Peige Song, Yan Zhang, **Jinyue Yu**, Mingming Zha, Yajie Zhu, Kazem Rahimi, Igor Rudan, 2019. Global Prevalence of Hypertension in Children: A Systematic Review and Meta-analysis. *JAMA Pediatrics*, 173(12), 1-10.
11. Peige Song, **Jinyue Yu**, Igor Rudan, 2018. Prevalence, risk factors and burden of diabetic retinopathy in China: a systematic review and meta-analysis. *Journal of Global Health,* 8(1).

Conferences (poster presentation)

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Abbreviations

ACE Abundance-based Coverage Estimator
BAI Beck Anxiety Inventory
BCH Beijing Children's Hospital
BEBQ Baby Eating Behaviour Questionnaire
BM breast milk
BMI body mass index
BP blood pressure
CG control group
CI confidence interval
CNS central nervous system
CS caesarean section
DBP diastolic blood pressure
DC dendritic cell
DR-SSC delivery room skin-to-skin contact
EBF exclusive breast feeding
EMT entero-mammary trafficking
ET early term
FT fingertip temperature
GA gestational age
GC glucocorticoids
GI gastrointestinal
HMO human milk oligosaccharide
HPA hypothalamic–pituitary adrenal
HR heart rate
IG intervention group
IS infant stool
IIFAS IOWA Infant Feeding Attitudes Scale
LP late preterm
MS maternal stool
MDD major depressive disorders
NMDS Non-Metric Multi-Dimensional Scaling
NICU neonatal intensive care unit
OTU operational taxonomic units
PASS Perinatal Anxiety Screening Scale
PCR polymerase chain reaction

PMR progressive muscle relaxation
PSS Perceived Stress Scale
RDS respiratory distress syndrome
SAD seasonal affective disorder
SBP systolic blood pressure
SCN suprachiasmatic nucleus
SD standard deviation
SDS standard deviation score
STAI State Trait Anxiety Inventory-State
T2DM type 2 diabetes
UNICEF United Nations Children's Fund
VAS visual analogue scale
WHO World Health Organization

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Chapter 1

General Introduction

This chapter consist of two sections. The first provides a general background and rationale for my PhD project, including an overview of the importance of breastfeeding and early infancy, current issues in breastfeeding worldwide and in specific population groups. The second section outlines a brief description of each chapter of this thesis. Detailed background and rationale for this research are provided in the next chapter.

1.1. Background of the research

Early infancy is a critical period of development and has an important impact on long term health and development. Early life nutrition therefore presents a window of opportunity where the infant's health can potentially be improved[1]. Breast milk is a natural first food and an ideal nutrition for the newborn. Increasing evidence shows the effects of human breast milk on optimising infant growth and development, as well as protecting against infection and developing the immune system [2-5]. Exclusive breastfeeding (EBF), defined as the practice of only feeding an infant with mother's breastmilk (ideally for the first six months of life with no other food or water added), has the single largest potential impact on child mortality of any preventive intervention [6]. However, despite a number of health programmes designed to promote breastfeeding, it is widely recognised that the EBF rates in many countries are disappointingly low and resistant to change [2]. Overall, less than half of women globally exclusively breastfeed their infants up to six months, with a rate of 39% reported by United Nations Children's Fund (UNICEF) in 2012 [7]. EBF is even harder for mothers who deliver a preterm infant for a number of reasons, including stress experienced as a result of separation when the infant requires intensive care combined with concern about the infant's wellbeing and the lack of breast stimulation by an immature infant who may be too small or sick to breast-feed [8]. A recent cohort study conducted in Beijing, China showed that the 6-month EBF rate was only 22.5% (63 of 280 preterm infants) and that a higher level of breastfeeding self-efficacy was a significant predictor of EBF [9].

While a number of attempts to improve breastfeeding rates focus on providing additional support, many aspects of the breastfeeding process remain poorly understood. Apart from the socio-economic and cultural factors which may influence a mother's decision on breastfeeding, lactation performance is also influenced by maternal physiological and psychological condition, as well as infant behavioural factors [10]. It should be noted that breastfeeding is a dynamic process which involves complex physiological signalling and behavioural negotiation between the mother and the infant. As suggested by parent-offspring conflict theory, each offspring is selected to demand more resources than the mother is selected to provide [11]. In other words, mothers may invest less than would be optimal for the infant, which will still enable the offspring to survive and reproduce in future life [10, 12]. This interaction may restrict maternal resources invested in the current offspring and for humans, it could influence the maternal lactation strategy. These processes may shape infant behaviour and feeding, including appetite regulation, and hence may also influence infant growth. Biological signalling is thought to involve bioactive factors (e.g. hormones) in breast milk, but is largely unexplored in human studies.

The beneficial effects of breastfeeding are even greater for preterm infants, with improvements in host defences, absorption of nutrients, gastrointestinal function, and neuro-developmental outcomes [13, 14]. Compared to mothers who have healthy term infants, mothers of preterm babies experience more difficulties in establishing breastfeeding [9], and therefore have greater tension in the mother-infant relationship during lactation [8]. This may lead to greater postpartum stress and affect the health

outcomes of mother-infant pairs. In China, mothers who have infants born at or before 37 weeks gestation often experience mother-infant separation as the infants are normally cared for in the neonatal intensive care unit for the first 1-3 days after birth. Evidence shows that maternal separation is an early life stressor that can result in long-term increases in hypothalamic–pituitary adrenal (HPA) axis activity, which is the main stress axis in mammals [15, 16]. Hence, whilst mothers of late preterm (LP, 34 0/7 – 36 6/7 weeks gestation) infants and early term infants (ET, 37 week gestation) are physiologically able to establish breastfeeding, lactation outcomes in this population are still poor in China as in many countries [17].

The effects of relaxation therapies on the reduction of maternal postpartum stress have been well-demonstrated [18]. Compared to mothers of healthy term infants, there is greater stress/increased tension during lactation for preterm mothers. Several relaxation methods, such as visual imagery [19], verbal protocols [20], and music therapy [14, 20], have shown beneficial effects on increasing the breast milk volume in preterm mothers. However, the quality of such studies is limited by weak study design, high attrition rates, and/or small sample sizes. Moreover, mechanisms of the relaxation effects on preterm mothers were under-investigated in existing studies. Hence, the primary objective of this research is to explore the effects of a relaxation intervention on reducing maternal stress and improving infant growth during the early postpartum period in mothers who are breastfeeding their LP or ET infant. I first provide a comprehensive literature review about human lactation and the interaction between mother and infants from psychological, anthropological, and microbiological

perspectives. Next, I describe a pilot study conducted to evaluate the most suitable relaxation intervention for the study population. Following this, a randomised controlled trial (RCT) was conducted to assess the effects of the relaxation intervention on maternal psychological changes, infant growth, and changes in breast milk and gut microbiota among LP and ET mothers and infants. This research addresses an important practical problem in mothers of LP and ET infants, and the results of this study will provide greater understanding about maternal-infant factors which influence the success of breastfeeding, and which may then be useful targets for future interventions.

1.2. Overview of the thesis

This thesis consists of eight chapters. A brief description of each chapter is provided below:

Chapter 2: This chapter provides a literature review of topics relevant for the research described in the thesis, starting with an introduction of mother-infant signalling during breastfeeding, followed by a discussion of parent-offspring conflict. Then, the chapter reviews the literature on mother-infant signalling from physiological, psychological and anthropological perspectives and evaluates previous intervention studies using relaxation therapy on preterm mothers. The reason for choosing LP and ET mother-infant pairs as the target population for the research is also outlined, and the innovative concept of the role of microbiome and HMO in mother-infant signalling is introduced. This chapter ends with a summary of current knowledge and research gaps, followed by

the plan of the pilot study and the RCT - Breastfeed a better youngster (BABY)- the BABY study.

Chapter 3: provides details of the pilot study, which was designed to select the most suitable relaxation intervention for the subsequent RCT. Five relaxation interventions were evaluated. Potential mechanisms for the influence of stress on breastfeeding outcomes are also discussed in this chapter.

Chapter 4: outlines the research methodology of the BABY study, including the research hypotheses, study design, outcome measures and study procedures. This chapter also describes the methods used for data collection and planned statistical analyses.

Chapter 5: provides descriptive characteristics and socio-demographic background factors of the population of the BABY study, and a comparison with mothers in Beijing and mothers in the general Chinese population.

Chapter 6： presents the findings of the primary and secondary outcomes of the BABY trial. Results are discussed in the context of published papers and interpreted from physiological, psychological, and anthropological perspectives. Strength and limitations of the RCT are also discussed.

Chapter 7: This chapter provides primary results of the microbiome analysis of the BABY study. Due to the COVID-19 pandemic, government policy in China was changed, and

the complete data from this analysis could not be released at the time of writing as the necessary approval is still pending, but available data are presented and discussed.

Chapter 8: This chapter summarises the findings of research, including the pilot and the BABY studies. An interpretation of the study results is provided. Strengths and limitations of the research are discussed, followed by implications and suggestions for practice, policy and future research.

Chapter 2

Literature Review

This chapter starts with an overview of breastfeeding in the general population and in mothers following late preterm and early term delivery, followed by an introduction of human lactation from evolutionary perspectives. Then, the concept of mother-infant signalling is introduced and interpreted from physiological, psychological, and anthropological perspectives. Moreover, the novel concept that microbiome factors played in mother-infant signalling is introduced. At the end of this chapter, I summarise current research gaps and limitations in existing experimental studies, followed by the rationale for the proposed study.

2.1. Overview of Breastfeeding

Human breast milk is the most ideal food for infants and breastfeeding is important for an infant's health and survival. As an ideal nourishment for the infant, breast milk alone can provide all the required nutrients, including vitamins and minerals, for the first six months of life [21]. The World Health Organization (WHO) and UNICEF recommend mothers to put neonates to the breast within one hour of birth, breastfeed infants exclusively for the first six months and continue breastfeeding for two years and beyond, together with complementary feeding starting in the sixth month [21, 22]. The short- and long-term medical and neurodevelopmental advantages of breastfeeding have been well documented. Studies showed that early initiation of breastfeeding, preferably within the first hour after birth, can significantly reduce overall neonatal mortality [23, 24]. Moreover, longer breastfeeding is associated with reduced risk of diarrhoea and acute respiratory infections, fewer dental malocclusions and higher intelligence, compared to no breastfeeding or shorter breastfeeding periods [2, 25-28]. Additionally, studies suggested potential effects of breastfeeding on preventing future obesity and type 2 diabetes (T2DM) [29]. Apart from this, breastfeeding also benefits the mother. Growing evidence shows that breastfeeding can protect the mother from breast cancer, postpartum stress and negative mood; it could also help to prevent future risk of T2DM and ovarian cancer [28, 30-33]. A systematic review suggested that the scaling up of breastfeeding could prevent approximately 823 000 child deaths and 20 000 breast cancer deaths every year [2].

WHO set “EBF under 6 months” as an indicator for studies assessing infant and young child feeding practices; the ‘EBF’ rate refers to the proportion of infants 0–5 months of age who are fed exclusively with breast milk (received only breast milk during the previous day) [34]. Under this definition, breastfeeding by a wet nurse and feeding expressed breast milk are accepted, however UNICEF data show that in low-income and middle-income countries, only 37% of infants are exclusively breastfed within the first 6 months of age, and the duration of breastfeeding is even shorter in a number of high-income countries [2, 7]. Compared to term infants, the incidence and duration of breastfeeding in preterm infants remains lower. In the United States, while the breastfeeding initiation rate of term infants was 69% in a large national survey [35], the average rate of breastfeeding initiation for preterm infants was approximately 50% as reported in a body of small cohort studies [36-38].

Apart from term infants, there are specific challenges to breastfeeding for mothers who deliver a preterm infant, regarding establishing and maintaining a milk supply and transitioning from gavage feeding to breastfeeding [36]. Preterm has been defined as delivery prior to 37 completed weeks of gestation [39]. In clinical settings, mothers of most preterm infants have to pump breastmilk for weeks or months until their infant is able to feed directly from the breast [40]. Particularly, the “within-preterm” classifications have evolved over time, because “34 weeks of gestation” marks a turning point in obstetric management after which antenatal steroids are not typically recommended [41]. Hence, the descriptor “near term” was used for infants born between 34 and 37 weeks gestation [42]. However, in 2005 the US National Institute of

Child Health and Human Development workshop changed the designation of deliveries between 34 0/6 0 to 36 6/6 weeks from “near term” to “late preterm” to emphasize that these infants experience morbidities and mortality similar to those of preterm infants rather than term infants [43]. This new terminology aims to properly communicate that late preterm (LP) infants are immature and therefore vulnerable to complications in the immediate neonatal period, including the regulation of breathing, temperature control [44], glucose and bilirubin metabolism [45], alert-wake behaviour, and effective breastfeeding [46].

Compared to preterm infants born before 34 weeks, in clinical settings, LP infants are often cared for in the general maternity setting, using breastfeeding guidelines and interventions that are designed for term infants [46]. The reason for these practices in many countries is that healthy late preterm infants are more similar to term infants than preterm infants who are born before 34 weeks gestation. Therefore, LP infants are often considered as “not really premature” or “almost full-term.”[46]. Due to this, health professionals prefer to use baby-friendly approaches to support breastfeeding, without either supplementation or pharmacological interventions for mother or infant. Although this can protect some LP infants from overtreatment, studies observed that mothers of LP infants are more likely to experience unsuccessful establishment of lactation and are especially susceptible to early breastfeeding failure [46-48], including early breastfeeding cessation, parental feeding-related anxiety, and re-hospitalization for feeding issues [49]. For LP mothers, the breastfeeding rate was much lower than mothers of term infants, with figures of 82.0% and 75.4% (p=0.012) in term and LP

infants respectively at 6-weeks in the UK 2010 Infant Feeding Survey [50]. A cohort study (n=2977) comparing breastfeeding difficulties and exclusive breastfeeding between late preterm and term infants showed that mothers of LP infants were less likely to report exclusive breastfeeding at 4 months compared to term infants (OR 0.67, 95% CI 0.46–0.97) [51]. Meier et al suggested LP infants often lack the ability to consume an adequate volume of milk at the breast, and their mothers are at risk for delayed lactogenesis [46]. Despite this, less research has focused on this population due to its relatively "healthy" status compared to those born less than 34 weeks' gestation.

Similar to LP infants, increasing evidence has shown that feeding difficulties are common in infants born between 37 to 38 weeks of gestation, compared to those born at 39 weeks or greater [42, 49, 51, 52]. The report of a WHO workshop in 2012 recommended that births occurring between 37 0/7 and 38 6/7 gestational weeks be designated as "early term" (ET) [42]. Although it was suggested that both LP and ET infants are born prior to the maturation of many body organs and systems and are especially protected by the bioactive components in human milk [53-55], mothers of those infants are less likely to initiate breastfeeding and less likely to continue breastfeeding after discharge [49, 55]. Additional research demonstrates higher risk of rehospitalisation for feeding-related issues in early term infants, compared with full term infants [52], suggesting a relative physiologic immaturity that may have important implications at a population level. Moreover, infants born at 37 weeks may have a higher risk for behavioural problems compared to infants born from 38 weeks onwards; the Western Australian Pregnancy Cohort Study (n=2900) found that infants born at 37 week's gestation were

at increased risk for overall and externalising behavioural problems, while LP infants and infants born at 38 weeks did not show a significantly increased risk for behavioural problems [56].

Additionally, infants born at 37 weeks gestational age (GA) have a higher risk of respiratory distress syndrome (RDS) compared to other full-term infants. A case-control study including 615 term infants (205 with RDS, 410 without RDS) found that infants<38 weeks GA had a higher risk of RDS compared to those born at 38 weeks onwards ($p<0.001$). This study was conducted in Bayi Children's Hospital in Beijing, which is one of the collaborating hospitals for my research, and has the largest neonatal intensive care unit (NICU) in China. When I conducted a clinical placement in this hospital, I found that infants born at 37 weeks were treated as LP infants in the Preterm NICU, while infant ≥38 weeks gestational age (GA) were cared for in the Term NICU and infants ≤33 weeks were in the Very Preterm NICU. This classification is based on their clinical experience that infants born at 37 weeks gestation may be more vulnerable than infants with greater GA. Further, Ray et al. investigated 6.6 million hospital discharge records of infants born in California between 1993 and 2005 and found that 35-, 36-, and 37-week presented higher risk of rehospitalisation for feeding-related issues compared with term infants [57], suggesting that the relative immaturity of LP and ET infants was associated with higher risk of health complications and feeding difficulties after birth, and breastfeeding can be more challenging for mothers of those infants.

Thus, current evidence showed that both health-related problems and breastfeeding difficulties are greater in both LP and ET infants, with worse outcomes for infants born at 37 weeks compared to 38 weeks. Due to these, studies suggested that mothers of preterm infants, either early or late preterm, presented more distress than mothers of full-term infants [58-60] Hence, it is possible that relaxation therapy might therefore be more effective.

2.2. Evolutionary and Anthropological Aspects of Lactation

The process of lactation was gradually established among a group of animals called synapsids, long before the emergence of mammals approximately 300 million years ago. This process involved the secretion of an antimicrobial liquid from epidermal glands, serving to protect and moisturise their eggs. In other words, the original purpose of lactation was microbe-killing rather than for nourishment [61, 62]. It has been hypothesised that when, approximately 200 million years ago, the Mammalia class evolved from the Therapsida, this epidermal or apocrine-like gland gradually evolved into a mammary gland that secreted a liquid containing immunologically active substances. This liquid is known as milk [62, 63]. At this stage, the mammalian milk still worked as a protective fluid, harbouring antimicrobial proteins predominantly for the protection of the offspring; the nutrition functions developed later [64]. Originally, many components of milk had dual roles, working synergistically both to protect and nourish the infant mammal. These components are specific to the species, as well as the offspring's characteristics, in order to ensure optimal compromise between maternal

and offspring interests in order to promote growth, development and survival [62, 65]. In general, highly concentrated milk is produced by animals that grow fast during the postnatal period and have short life spans, as higher energy is needed for accelerated growth [66, 67]. For example, the milk of the hooded seal has the highest percentage of fat (61%) of all mammals, due to the immediate need for a significant amount of stored fat for insulation and thermogenesis[68]. As a result of the short lactation period, high energy is needed to sustain the pups post-weaning, before they establish their swimming skills and are capable of hunting for food. In comparison, primates, including humans, have a slower growth rate during infancy and their milk is relatively dilute, with a low concentration of protein and fat. This suits the longer period of primate infancy. The duration of lactation is also longer and maternal bodily reserves are not depleted so quickly due to their offspring's slower rate of growth and development [66, 67].

Nevertheless, within species, milk production also varies among mothers. In humans, for example, it is increasingly apparent that maternal factors such as body composition, diet, ethnicity, geography, genetics and lifestyle all contribute to the unique milk signature of each woman [64, 65]. For instance, Bzikowska-Jura et al. observed that maternal adiposity was related to HM protein and energy content at three months' lactation, irrespective of diet [69]. The breast milk fatty acid profile [70] and some micronutrients in breastmilk are also found to be influenced by various components of the maternal diet, such as iodine [71], vitamin A [72] ,vitamin C [73, 74] and vitamin D [75]. Moreover, a recent study showed that concentrations of the immune active molecules transforming growth factor-β2, immunoglobulin A, and hepatocyte growth

factor in human breast milk were higher in African women than in Italian women, suggesting a stronger response to the environment and thus greater infant protection against infection [76].

Moreover, traces of the evolutionary process in human lactation are apparent in the presence of immune cells in human milk that increase significantly in response to both maternal and infant infections. Twigger et al. [77] have identified antimicrobial proteins, granulysin and perforin along with other granzymes released by leukocytes in human breast milk, that are elevated due to maternal breast infection. Immune cells found in breast milk may therefore offer protection against infection for both the infant and the breast. Studies show that human breast milk protects against diarrhoea and infection in infants, reduces the risk of obesity, type 2 diabetes and cardiovascular disease later in life, and is associated with an increase in cognitive development [2, 78, 79]. It has also been reported as decreasing the risk of breast and ovarian cancers in mothers [2, 80]. The Developmental Origins of Health and Disease Hypothesis recognises that the breastfeeding phase, which can continue up to two years and beyond, plays a major role in the continuum of programming of the lifelong health and development of the infant [81, 82]. Early life nutrition therefore presents a window of opportunity where the infant's short and long-term health can potentially be improved in the face of escalating rates of chronic disease that have reached epidemic proportions [64].

In addition to maternal factors, lactation performance (including milk synthesis and composition) is also influenced by the communication between the mother and the

infant during lactation, such as the pattern of 'demand' by the infant and how the mother responds, [10]. This signalling during breastfeeding will be discussed in the next section.

2.3. Mother-Infant Signalling during Lactation

2.3.1. Overview

Mother-infant signalling can be used to describe the way in which infants and mothers communicate and interact during the process of feeding [83]; infants have a number of ways of expressing their needs, including vocalising, and mothers in turn respond by giving or refusing them access to their nipple – a reaction which impacts on the volume and quality of the milk the mother produces. Mother-infant signalling is a major feature of early parenting, particularly in the period immediately after birth, since mothers who are feeding their newborns have to learn how to understand the prompts and signals of their child in order to respond in a timely and appropriate manner. Numerous mammalian species share the same range of methods for recognising their child: either by their odour or their vocalisations, as well as the baby's body heat and through their sense of touch [84]. Taken together, these cues make sure that the mother will care for and safeguard their newborns and give them a suitable physical environment, and the stimulus they need to grow and develop. The young, for their part, become familiar with the mother and demonstrate a pattern of suckling which mirrors their awareness of the mother's odour, touch and the temperature of her body [84]. In humans, mother-infant signalling – particularly during breastfeeding – is a key inter-relational factor between

mother and infant during the newborn's early life, with the signals indicating hunger or repletion being among the most precise and obvious cues given by the neonate.

In this section, I firstly introduce the concept of parent-offspring conflict as the theoretical basis of the mother-infant signalling. Then, the mechanisms of mother-infant signalling are considered from psychological and physiological perspectives in section 2.3.3 and 2.3.4.

2.3.2. Parent-offspring conflict

Lactation is very energetically expensive, posing an additional cost of approximately 2.7MJ/d in the first six months; the daily energy expenditure of lactating women is 25%-30% higher than that of moderately active non-pregnant, non-lactating women of average size [85]. In the first three months of life, babies require between 500-680 kcal/day obtained from an energetic cost comprising milk energy density, gross composition and volume and efficiency of synthesis [86, 87]. Parent-offspring conflict is predicted to occur particularly during energetically costly periods such as lactation, which could influence the maternal lactation strategy. For mammals, when resources may no longer be sufficient to cope with the high energy needs of lactation, growth and replenishment of body reserves, a nursing mother may decrease the allocation of resources to current reproduction to ensure their own future reproduction and survival [88], as expected by Trivers' parental investment theory [11]. This has also been described as "trade-off decisions" where females may decrease nursing activities and increase time spent on foraging to maximise their own fitness and ensure future

reproduction [66, 86]. Meanwhile, their current offspring can express their needs by begging behaviour such as suckling vigour and vocalisation [89].

Based on Trivers' theory, parent-offspring conflict predicts that each offspring is selected to demand more resources than the mother is selected to provide [11]. As mentioned previously, lactation is very energetically expensive. Therefore, investing such high energy to maximise the fitness of the current offspring may lead to less investment for later offspring, thereby influencing the extent to which maternal genes are passed to future generations. Given this, mothers may restrict the resources invested in the current offspring and save more resources for future offspring since that will also benefit maternal genes [90]. In other words, mothers may reduce investment in their current offspring below the level that would be optimal for that offspring, as long as the offspring will be able to survive and reproduce in future life [10, 12]. Nevertheless, while offspring may require more resources than would be optimal for mothers to provide, the demand will not reach a level which can threaten their mother's health, thereby preventing them from receiving future resources [10, 90]. In this context, lactation essentially represents a conflict or tension between the supplier and consumer of breast milk during lactation period [89]. For example, the mother can allow or restrict nipple access and the offspring can demand feeding through vocalisation.

As a result of this conflict, during the process of evolution, mammals developed a number of reproduction and lactation strategies, which resulted in major disparities in the frequency and length of time dedicated to milk feeding – and the composition of the

milk itself. Over time, lactation has become adaptable and responsive to various elements of maternal phenotypic changeability. According to Jones, J.H. [91], primates tend to have slow-growing offspring, who depend on their parents to satisfy their needs for long periods of time. When compared with many other mammals, the nutritional value of the primates' milk transferred from the mother to the offspring is fairly low, and this has the advantage of ensuring that the energy cost of investment is spread over a longer period [92]. Nevertheless, it also potentially makes the period of mother-offspring conflict longer. Human lactation is shaped by further adaptations and, when compared to other primates, human milk contains high levels of oligosaccharides (HMO) [93]. A number of researchers have argued that this adaptation supported the rise in population density common to sedentary communities, by providing greater and more effective protection against infections [66]. The HMO may also be involved in the microbiota interaction between maternal breast milk and infant gut, which is further discussed in Section 2.4.

As suggested by Tully and Ball [12], the 'competition' for maternal resources largely occurs subconsciously. During the lactation period, infants can show their demands through a sequence of signals, such as their behaviour, appetite, vocalisation, or non-nutritive suckling. The mother's response can be shown by the signals of breastfeeding behaviour (e.g. skin-to-skin touch), the volume of milk produced, or the composition of nutritive or non-nutritive components in milk [10]. Hence, the optimal coordination between the new mother and her young involves a sequence of signalling on the part of

each that ensures that the young will be adequately cared for and show healthy physical, emotional, and social development [84].

2.3.3. Mother-infant signalling from a physiological perspective

The potential physiological signalling involved in breastfeeding includes changes in volume, nutrients, energy, and a number of non-nutritive factors in breast milk. Firstly, the mother could respond to infant signals by increasing breast milk volume, but could also restrict her supply by restricting nipple access, hence down-regulating milk synthesis and affecting milk volume [94] (Figure 1). Secondly, as shown in clinical and laboratory studies, the composition of macronutrients in breast milk also changes during a feed, which can have effects on infant responses, namely satiety [95]. Evidence shows that the concentration of fat in breast milk during a single feed is usually the lowest in the foremilk and highest (two- to three-fold) in the hind milk; and the energy content is 25–35 kcal/100 ml more in hindmilk on average than in foremilk [94, 96].Given this, the infant may respond by stopping suckling when they achieve satiation or feel full. This process of satiation could not occur in the same way in formula-fed infants, because they receive milk with a constant composition throughout the feed.

Moreover, satiety may also be modulated by certain non-nutrient components in breast milk, such as hormones or other endogenous substances. Studies suggest that mothers may have the ability to shape infant behaviour in early infancy by the transmission of biologically active compounds including the glucocorticoids (GCs, cortisol in humans) in milk during breastfeeding [97]. GCs are stress responsive steroid hormones that are

involved in stimulating and suppressing other stress-related hormones [98]. Certain stressful events activate the central nervous system and stimulate the hypothalamus to release corticotropin-releasing-hormone, which then causes the release of adrenal-corticotropin-hormone from the anterior pituitary eventually stimulating the adrenal glands to release cortisol[99]. In lactation, cortisol plays an important role in triggering lactogenesis II during parturition and early lactation [100]. Its effects during lactation include regulating tight junction permeability [101], as well as preventing apoptosis and the involution of breast tissue. Observational studies showed that elevated levels of cortisol in breast milk were predictive of negative temperament in infants [102-104]. Grey et al. reported that higher levels of GCs in human breast milk are associated with more negative infant temperament [102]. Consistent findings were reported by Glynn et al. [104] where higher maternal plasma GCs were associated with increased fearful temperament in breastfed infants, with no apparent association among non-breastfed infants. The results of these studies suggested that higher GC levels in mothers who were stressed can result in negative infant temperament, such as fear and sadness through breastfeeding practices. It can be hypothesised that once a breastfeeding mother is stressed, the elevated GCs in breast milk may lead to the expression of negative infant temperament; this may further down-regulate milk synthesis and affect milk volume, finally resulting in a 'vicious circle' between mother's breastfeeding practice and infant behaviour.

Apart from milk hormones, breast milk also contains a range of potential signal components, such as growth factors, cells, oligosaccharides, microbiota, and microRNA

[92]. All of these possible mechanisms of mother-infant signalling could be involved in the mother-infant conflict during breastfeeding, and these may take place at a more intense level during early life, when energy demands for growth are higher. However, current evidence on the role of such factors is limited, partly because most of the current studies in this field use an observational design, making it difficult to explore causality in the complex interrelationships between breast milk and infant behaviours. Another limitation relates to the practical issues during sample collection, including the method of obtaining representative samples of expressed breast milk and the timing of sampling; since the fat and energy content varies during the day and during the course of a feed [92, 94]. Appropriate milk sampling methods are imperative when examining variability of milk components. In this context, Bzikowska-Jura et al found a weak relationship between human milk fat content and maternal BMI using an intense sampling regime to account for changes in fat over the course of 24 h [69]. George et al also highlighted sampling as one of the major challenges when examining milk lipids [105]. Moreover, the breast milk samples used in Grey and Glynn's studies discussed above were taken at random times from a single feed and cortisol concentrations may vary within a feed or diurnally [106]. Hence, the question of whether there is indeed any relationship between GCs levels in breast milk and infant behaviour merits further investigation with a more robust methodological design.

2.3.4. Mother-infant signalling from a psychological perspective

Figure 2.1 outlines the interaction between maternal and infant psychological status during the breastfeeding period. On the one hand, maternal psychological state can influence milk ejection, often described as the let-down reflex [107], which is activated by the hormone oxytocin during the lactation period. Oxytocin, which is secreted by the posterior pituitary gland, is usually stimulated or triggered by infant suckling. If the mother is stressed, secretion of oxytocin can be suppressed. The let-down reflex can then be inhibited, leading to the disruption of milk flow and reduced milk volume, and finally resulting in milk ejection difficulties [107-110]. Alternatively, the let-down reflex can be stimulated when a mother intends to or expects to breastfeed, including thinking, hearing, touching and/or smelling her baby, or by any pleasurable experiences between mother and infant [111]. Regular skin-to-skin contact can facilitate breastfeeding [112]. On the contrary, if the mother is unwell or distressed, secretion of oxytocin can be suppressed, which can result in difficulty in milk ejection. As suggested by a number of studies, emotional distress in mothers inhibits the let-down reflex, leading to disruption of milk flow and reduced milk volume, hence affecting breastfeeding success[32, 113, 114].

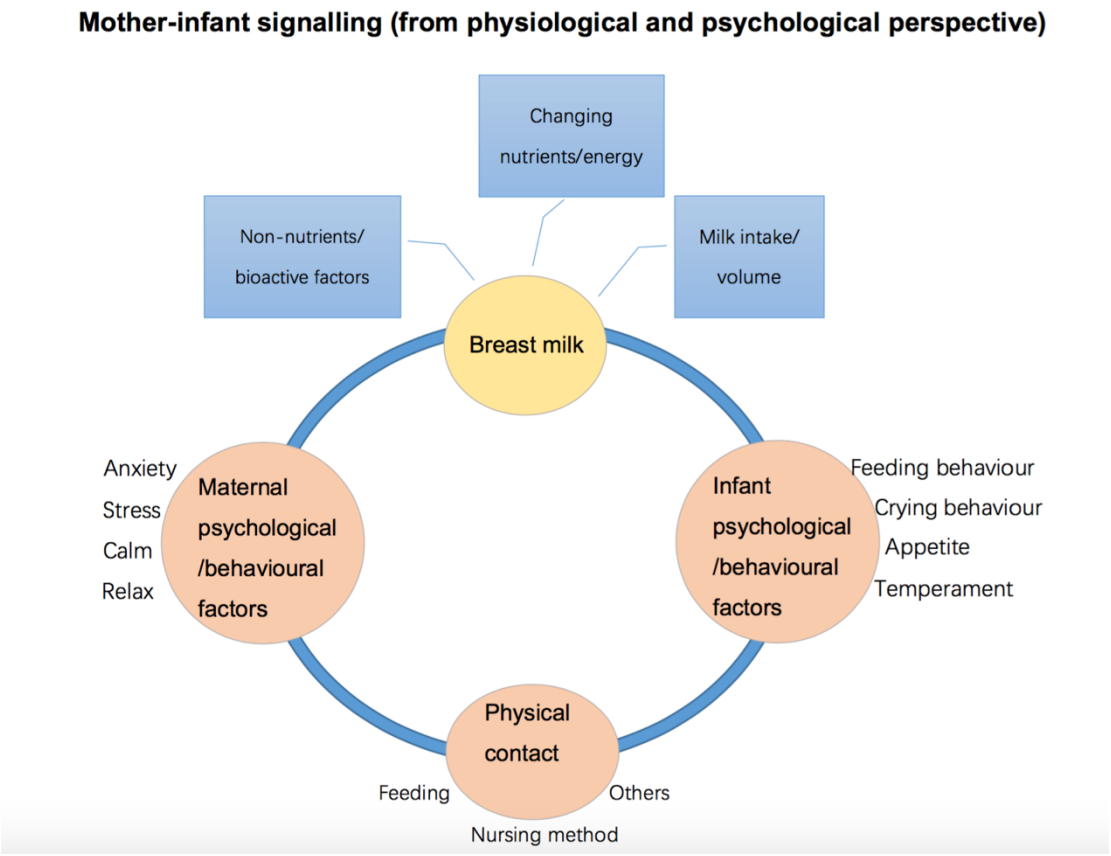


Figure 2.1. Possible mechanisms of mother-infant signalling

Moreover, evidence shows that mothers with psychological distress may be less sensitive to infant cues and can have difficulties in interacting with or responding to their babies including contact or touching [115, 116]. Thus, infants can be affected by the negative moods of their mother. Once the mother is stressed, the infant may spend more time on crying in order to receive more care or feeds from the mother; this can eventually result in increased maternal stress; meanwhile, since crying costs calories, the infant may require more energy from the mother, which leads to a vicious circle between mother and infant. A systematic review reported that infant distress and crying behaviour in the first three months is associated with the experience of tiredness and

fatigue in mothers and may trigger depressive symptoms and affect parent–child interaction [117]. However, most studies included in this systematic review used an observational design, which could not establish causal relationships between infant distress and maternal mental health. To further investigate mother-infant signalling, intervention studies with rigorous design are needed in future research.

2.4. Intervention studies investigating mother-infant signalling

The existing evidence that maternal psychological state could influence lactation success by affecting milk ejection suggests that milk ejection may also be improved by reducing maternal stress, for example by using relaxation interventions during breastfeeding. Relaxation techniques have been used in several areas, such as mental health, special education, rehabilitation and social development [14, 19]. Particularly, studies found various physiological effects of such techniques on reducing stress and anxiety, which were associated with improved endothelial function [118]. Although the mechanisms underlying the effect of positive emotions on endothelial vasoreactivity remain unclear, one possible link is endorphin-mediated activation of endothelium-derived nitric oxide, an effect opposite to that observed when the potent vasoconstrictor endothelin-1 is released in response to mental stress [119]. A number of studies showed that increased endorphin levels may lead to the release of lactogenic hormones and help increase breast milk production [14, 18, 20]. Thus, relaxation interventions may improve endothelial function, help the body produce and release nitric oxide and endorphins, reduce mental stress and tension, and increase breast milk production [120].

2.4.1. Relaxation interventions in mothers of term infants

Several experimental studies have examined the effects of relaxation techniques on maternal psychological state and breastfeeding outcomes [18]. However, most of them only examined maternal outcomes or infant growth outcomes, leaving mother-infant signalling under investigated [121-123]. Mohd Shukri et al conducted an RCT (the MOM study) investigating the effects of a relaxation meditation tape on maternal psychological state, breast milk intake, milk cortisol levels, infant behaviour and growth in Malaysian mothers [124]. A total of 64 primiparous breastfeeding mothers of healthy full-term infants were randomly assigned to relaxation (n=33) and control group (n=31) at 2-week postpartum. Both groups received standard breastfeeding support while the relaxation group mothers were asked to listen to a relaxation meditation tape at least once a day. Data were collected at 2-, 6-, 12- and 14-week after birth to measure maternal stress and anxiety, breast milk intake and milk cortisol, and infant behaviour and growth. Results showed that mothers in the relaxation group had significantly lower stress scores when compared to the control group and a greater reduction in milk cortisol concentrations during a feed when the mother was first exposed to the therapy. Moreover, significantly higher milk intake and milk carbohydrate composition were observed in relaxation group compared to control group at the 12-week home visit. The relaxation group also showed a non-significant trend of fat and energy increase at all home visits than the control group. Additionally, the author reported significantly higher infant weight and BMI gains, as well as longer sleeping duration of infants in the relaxation group. Findings of this study confirmed the effects of relaxation intervention

on maternal psychological state and infant growth. From an anthropological perspective, effects of the intervention suggested that by experimentally manipulating the maternal psychological state, mothers with reduced stress may invest more on their infants by optimising the milk composition, this may simulate infant sleep. Mothers who were relaxed may also produce more milk which may contribute to the higher milk intake in relaxation infants and resulted in higher weight gain. Moreover, from a psychological perspective, more relaxed mothers might sleep longer themselves, which may also affect the infant's sleep duration, considering all mothers and infants in the trial were co-sleeping.

Findings of the trial could also be interpreted from a physiological perspective. As explained in section 2.1, mothers who were less stressed could produce milk with reduced concentrations of GCs. Hence, the reduced GC concentration in breast milk of intervention group mothers may consequently result in prolonged infant sleeping time. However, the observed significant differences in milk cortisol within a feed between groups were only found at the first visit, suggesting the intervention may have been more effective in reducing cortisol concentrations when mothers were first exposed. The trial also reported some non-significant trends regarding the macronutrient content of breastmilk in relaxation-group mothers, however, the small sample size limited the statistical power to detect differences. Besides, the population included in this trial were mothers of healthy term infants who were well-supported by family; they may have been relatively "relaxed" at baseline, which could have limited the ability to see effects of the relaxation therapy on some assessed outcomes. As suggested by the author, it

would be worth investigating the use of this relaxation intervention in settings where breastfeeding is more challenging for mothers and the expected baseline stress might be higher.

2.4.2. Relaxation interventions in mothers of preterm infants

Mothers of preterm infants may experience higher stress levels than those with term infants, especially if their infant is unwell. Several intervention studies have investigated the effects of relaxation interventions in preterm mothers. A literature review was performed to identify studies investigating the effects of relaxation interventions on maternal stress and breastfeeding outcomes in mothers of preterm infants. Studies were searched in PubMed based on the following search strategy: "relaxation" OR "relax" OR "meditation" OR "music" AND "breastfeeding" OR "infant feeding" AND "maternal stress" OR "milk volume" AND "randomised controlled trial" OR "RCT". Moreover, the first ten pages of Google Scholar was searched using the key words "relaxation" "intervention" "breastfeeding" and "maternal stress". Only studies in English were included. After reviewing the title and abstract, the paper was included if it was an RCT assessing the effect of a relaxation intervention on mothers who were breastfeeding their preterm infant. **Table 2.1** presents six intervention studies included in the review. The relaxation interventions used in the included studies were: guided yoga meditation [19, 20, 125], progressive muscle relaxation (PMR) [125, 126], and music therapy [20, 120, 127]. Varisxoglu et al. investigated the effects of listening to music on breast milk production in 40 Turkish mothers with premature infants. Findings show that mothers

who received music therapy reported significantly lower anxiety and higher milk volume than control mothers. The cortisol level in breastmilk was significantly lower in post-intervention compared to pre-test states, although the milk production between groups was not significant. Similarly, Dabas et al. reported significant reductions in maternal stress and anxiety as well as a significant improvement in milk output in mothers who received the 10- day PMR training. The effects of relaxation therapy on milk volume were also observed in study conducted by Feher et al., which reported a 63% higher volume in relaxation mothers; and by Keith et al., in which the breast milk volume was significantly higher in mothers who received either verbal protocol only or verbal protocol plus other relaxation techniques, compared to the normal care group mothers.

Table 2.1. Summary of the intervention studies using relaxation therapy in preterm infants.

Study	Randomisation	Participants	Intervention/control groups	Outcomes and measures	Main results
Feher et al. (1989) **USA**	Not specified	Breastfeeding mothers of mixed parity (n=53); Preterm infants in the NICU for at least 10 days.	IG: A 20 mins audio cassette tape based on relaxation and visual imagery techniques. Listen once a day prior to Breastfeeding for 7-13 days. CG: Normal	Milk volume of single expression at 1-week after enrolment Milk fat: creamatocrit	Milk volume IG:90.01± 60 ml; CG:55.4±48.2ml; 63% higher in the intervention group, p<0.05.
Vianna et al. (2011) **Brazil**	A table of random numbers	Breastfeeding mothers (n=94); Preterm infants weighting ≤ 1,750 g who were not critically ill.	IG: 60 minutes music therapy three times a week for 60 days. CG: Normal care	Breastfeeding frequency and breastfeeding rate	Breastfeeding rates significantly higher in IG at 7-15 days after discharge (IG 88% vs. CG 70%, RR=1.26, 95%CI:1.01-1.57, p=0.03).
Keith et al. (2012) **USA**	Random schedule	Breastfeeding mothers of mixed parity (n=162); Preterm infants in the NICU or critically ill.	A: Control with normal care; B: Verbal protocol (12 mins) + Iullabies;	Milk volume: Number of times pumped and volume of milk produced; Milk fat: Creamatocrit collection of 1ml sample of composite	Milk volume at 14 days: Group B: 591.4±47.6ml, Group C:1028±48.8ml, Group D: 861.7±52.2ml. Control Group: 318.2±47.1ml. All p<0.001

			C: Verbal protocol + guitar music background + images of the infant; D: Verbal protocol only Use as often as possible while pumping milk for 14 days	breast milk of expressed milk, collected from day 1-14	Fat content at 11 days: Group B: 52.5±9.8, Group C:81.0±10.1, Group D: 56.3±11.2. Control Group: 46.5±10.0. Group C was significantly higher than control (p=0.016)
Karbandi et al. (2017) **Iran**	Toss of a coin	Breastfeeding mothers in Mashhad, Iran (n=60); Preterm infants with 32-36 GA	IG: Guided PMR (30-45 minutes) at least once a day for 2-month. CG: Normal care with relaxation breathing techniques	Breastfeeding self-efficacy, using Dennis breastfeeding self-efficacy standard questionnaire	The mean score of self-efficacy: 4-weeks: IG 50.51±6.79 vs. CG 44.63±6.35, p=0.001 8-weeks: IG 57.62±6.22 vs. CG 47.41±7.91, p<0.001
Dabas et al. (2019) **India**	Total enumeration sampling technique	Breastfeeding mothers (n=57); Preterm infants (26-33 GA)	IG: Administered yoga relaxation audio (including PMR) assisted relaxation technique followed by every day practice for 10 days; CG: Normal care	Maternal stress, assessed by Parental Stress Scale; Maternal anxiety, assessed by Perinatal Anxiety Screening Scale; Milk volume: Expressed milk of both breasts in	Significant reduction observed in maternal stress (2.9 ± 0.5 vs. 3.6 ± 0.6) and anxiety scores (19.8 ± 6.7 vs. 28.18 ± 11.7, p ≤ 0.05) and improvement in milk output (69.2 ± 19.3 vs. 54.1 ± 22.5, p ≤ 0.05) in IG compared to the CG.

				24hour divided by frequency of milk expression.	
Varisxoglu et al. (2020) **Turkey**	Not specified	Breastfeeding mothers (n=40) Preterm infants (mean GA=32.21±2.26)	IG: listened to music for 15 minutes across two sessions of milking with a pump at 11:00–16:00 on the second, third, and fourth days, and the amount of breast milk produced was recorded on the follow-up form; CG: underwent two sessions of milking without music. For four days.	Milk volume, expressed by pump; Salivary cortisol level Maternal anxiety assessed by Spielberger's State-Trait Anxiety Inventory (STAI)	Milk volume: Changes from third to fourth day in IG was significantly higher than CG (p=0.001); The final STAI-A mean score of the IG was significantly higher than CG (IG:46.2±3.5 vs. CG:40.1±6.1, p < 0.005), indicating a reduction in anxiety scores of the mothers who listened to music; Cortisol level: IG: pre intervention:5.58±2.78 nM post intervention: 4.42±1.69 nM (p=0.01) CG:pre intervention: 5.43±2.81nM post intervention: 6.13±3.22 nM (p>0.05) Breast milk production (3-4 days): IG 23.1 ±14.1 vs. CG: 12.3±11.8, p=0.001

Notes: PMR= progressive muscle relaxation; IG=intervention group; CG=control group; RR=relative ratio; CI=confidence interval

Although the sample size of these RCTs was adequate to detect hypothesised differences in the primary outcome(s) between groups, there were certain limitations that should be noted. Firstly, none of these studies indicated whether the randomisation schedule was prepared by an independent person and the randomisation method was not reported in Feher et al and Varisxoglu et al. Secondly, mothers in the control group were aware of the relaxation therapy treatment of the mothers in the intervention group. Therefore, there was a possibility that mothers in the control group might seek similar therapy during the study period—and this concern was not acknowledged in any of the RCTs. Moreover, the follow-up period in Vianna et al and Karbandi et al was 2 months, while in other RCTs the follow-up period was around 4-14 days, which could not allow evaluation of the effects of relaxation intervention after long-term, regular use. As identified by a previous study, the relaxation intervention may be more effective when mothers were first exposed to the intervention [124]. Hence, the positive results in these studies should be treat with caution.

In addition to these issues, the existing RCTs included in the present review only assessed the effects of relaxation intervention on milk production and maternal psychological status, without an assessment of effects on infant behaviour or growth. Besides, current RCTs using relaxation therapy did not discuss mother-infant signalling during breastfeeding, or consider whether the infant behaviour could in turn influence maternal breastfeeding practice and psychological status. Moreover, the breast milk sample collection methods in most existing studies were inconsistent, which can make

the results between studies less comparable. More importantly, due to the non-systematic search strategy used for my review, some relevant studies may not have been identified and the results might therefore be biased. Considering the time scale of my PhD project, it was not realistic to perform a formal systematic review prior to the main study. Although it is possible that the non-systematic search might have resulted in the exclusion of studies which report no effect of relaxation interventions on breastfeeding outcomes, the consistent results from the six included studies at least provided evidence on the beneficial effects of relaxation intervention. The present review also illustrated limitations of the included studies and revealed the current research gaps. Further studies with a larger sample size, especially those investigating both maternal psychological status and infant behaviour and growth outcomes with standardized procedures for breast milk sample collection are needed.

2.5. Novel view of mother-infant signalling from the microbiology perspective

2.5.1. Overview of microbiota, HMO, and intestinal colonization

The human body contains a diverse and sizeable community of microbial cells and genetic material, known as the microbiome. The microbial community inhabiting the gastrointestinal tract is characterized by its high population density, wide diversity, and complexity of interactions [128]. The number of microorganisms inhabiting the GI tract has been estimated to exceed 10^{14}, which encompasses about 10 times more bacterial cells than the number of human cells and over 100 times the amount of genomic content

(microbiome) as the human genome [129, 130]. The microorganisms that reside in the human GI tract exert a major effect on host health and disease, which is most marked in the development of basic physiological process [131]. The early microbial colonization of the neonatal intestinal tract commences at birth when delivery exposes the infant to a complex microflora. A comprehensive review showed that the initial microbiome in the infant's gut plays a vital and active role in infant development, and also has a maternal signature. Several studies in this review reported that the microbiota and HMOs in human breast milk have a profound influence on the early microbial colonization of the host [132].

As mentioned previously, HMOs are complex glycans that can nourish health-promoting bacteria particularly *Bifidobacteria* in the GI tract of breastfed infants [133-136]. Comprehensive reviews show that HMOs can also help to defend against pathogens by preventing adhesion to the gut epithelium when the immune system is immature during the early stages of life [137, 138]. In addition, the microbiota in mother's breast milk can be another important factor influencing the intestinal colonization of their children. Emerging evidence noted that breast milk can be a vehicle for transmission of gut microbiota from mother to neonate, and this vertical transfer of bacteria can contribute to the initial establishment of the microbiota in the developing infant intestine [139].

The following sections will discuss the effects of intestinal colonization and gut microbiome on modulating infant behaviour during breastfeeding, and consider whether the psychological status of the mother could affect the composition of

microbiota in breastmilk and thus influence the infant's behaviour by modulating the microbiota composition in the infant's gut (**Figure 2.2**). This could be another potential mechanism for physiological signalling during breastfeeding.

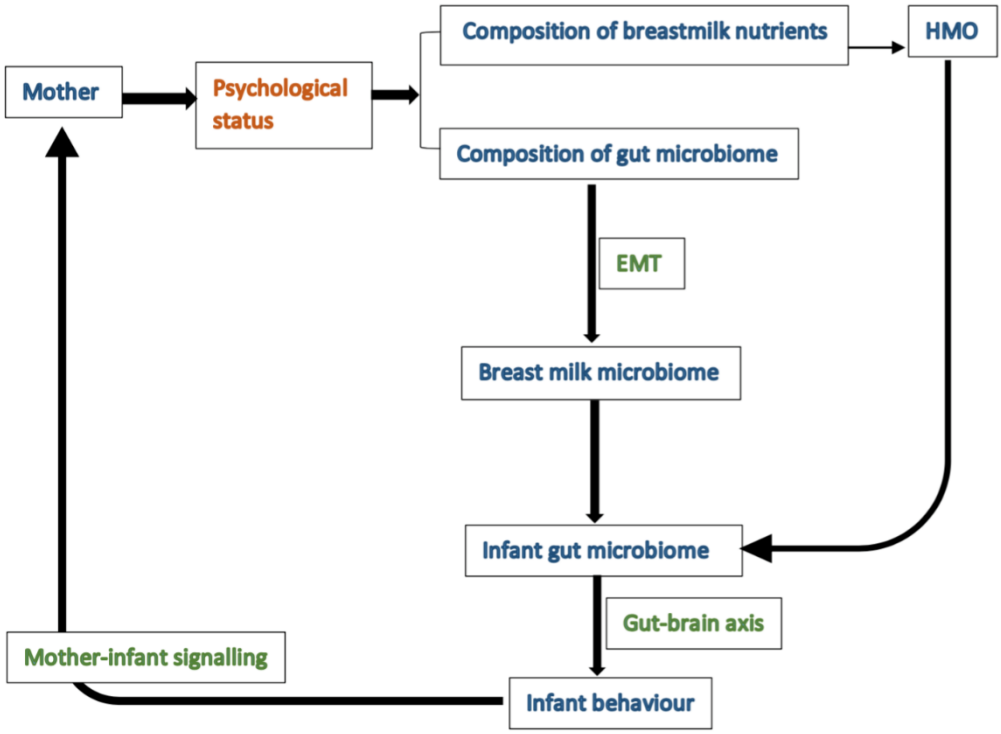


Figure 2.2. Hypothesis of mother-infant signalling through the microbiome and HMOs
Notes: HMO=human milk oligosaccharides; EMT= entero-mammary trafficking

2.5.2. Gut-brain axis: the bidirectional communication between gut and brain

The initial construct of gut-brain axis describes the complex bidirectional communication system linking the central nervous system (CNS) and the GI tract [132,

140]. The microflora residing in the GI tract communicates with the CNS, possibly through neural, endocrine and immune pathways, and thereby influences brain function and behaviour [141]. A common method used to investigate the interactions between the microbiota and the gut-brain axis is to compare germ-free animals with those colonized with a single strain or multiple strains of bacteria [142]. Several animal studies reported that the gut microbiota can affect immune activation, vagus nerve signalling, alterations in tryptophan metabolism, production of specific microbial neuroactive metabolites and bacterial cell wall sugars through the gut–brain axis [132, 141]. These findings suggest that modulation of the microbiota community could be associated with alterations in behaviours.

2.5.3. Mother-neonate transfer of maternal gut microbiota

It has been reported that the developing foetus is effectively sterile up until birth with microbial penetration of the amniotic space seen as an extremely rare occurrence [131]. During the delivery process and rapidly thereafter, microbes from the mother and surrounding environment colonize the gastrointestinal tract of the infant until a dense, complex microbiota develops [143]. Successful colonization and the structure of the infant microbiota community can be affected by a number of factors, such as the mode of delivery and feeding strategy. Studies comparing the maternal stool microbiota with that of the breast-fed or formula-fed infant suggested that infants who are breast-fed have a microbiome more similar to that of their mother than formula-fed infants [144].

Among all of the influencing factors, breastfeeding plays a key role in intestinal colonization and can be an important factor that influences the microbiota community structure of the infant's gut [131]. Recently, the use of culture and molecular techniques have revealed the dominance of *Staphylococci*, *Streptococci*, *Lactic acid bacteria* (LAB) and *Bifidobacteria* in human breast milk, and their role in the colonization of the gut microbiota of breast-fed infants [145, 146]. Clinical studies show that the composition of the gut microbiome differs between breast-fed and formula-fed infants, where a more stable bacterial population was observed in breast-fed infants and more diverse in formula-fed infants [147-149]. Besides, a recent study using the 16S RNA gene sequencing technique has characterized the bacterial communities present in milk samples of lactating women and faecal samples of their breast-fed infants [139]; results revealed that a number of typically gut-associated genera were common to both human milk and infant faeces, including *Bifidobacterium*, *Bacteroides*, *Enterococcus*, *Lactobacillus*, *Clostridium*, *Coprococcus*, *Escherichia-Shigella* and members from the *Lachnospiraceae* family. This finding is in agreement with previous studies [146, 150-153], suggesting that human breast milk can be the source of hundreds of bacterial phylotypes to the infant gastrointestinal tract and there is a mother-infant transfer of the detected bacterial genera through breastfeeding. Hence, it would be important to have a better understanding of the origins of the microbiota in breast milk and how it is transferred to neonates via breastfeeding. A hypothetical model to explain how some maternal bacteria could be transferred to the neonatal gut through breastfeeding is shown in **Figure 2.3**.

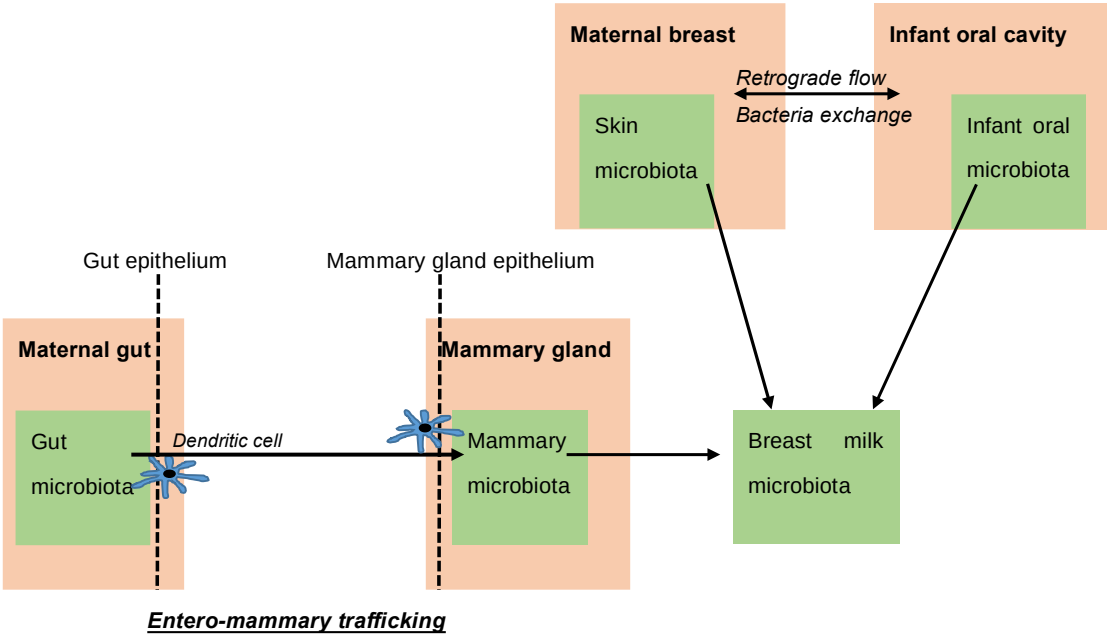


Figure 2.3. Hypothetical model for the origins of microbiota in breast milk

2.5.4. Origins of microbiota in breast milk

The breast milk microbiota could be obtained via three routes: the maternal skin, retrograde flow from the infant's oral cavity, and movement of microbiota from the maternal gut to the mammary gland [139, 144] (**Figure 2.3**). For the first route, molecular approaches have been employed to genetically type gram-positive organisms from the maternal skin, breast milk, and their infants to identify the commonality of specific strains in the dyad. Results suggest that skin bacteria may be transferred through the process of breastfeeding from the mother's nipple and surrounding areolar region to the infant [154, 155]. Hence, some of the maternal skin-associated microbiota,

such as *Staphylococcus*, *Corynebacterium*, and *Propionibacterium spp*, are introduced to the infant's oral cavity and enteric tract [156, 157].

For the second route, Ramsay et al [158, 159] applied the ultrasound imaging technique to observe the process of infant suckling. Results indicate that there may be a backward flow of breast milk from the infant's oral cavity through the nipple into the mammary gland, which provides an ideal route for the exchange of bacteria. This finding may explain the presence of some microbiota which have been noted in both the oral cavity of neonates and mother's breast milk, namely *Gemella*, *Veillonella*, *Staphylococcus*, and *Streptococcus* [160, 161].

For the third route, the entero-mammary trafficking (EMT) model [145, 155, 162] was proposed to explain the presence of typically enteric microbiomes identified in breast milk, such as *Bifidobacterium* and *Lactobacillus* [163]. Fernández and his research team confirmed the suggestions that the origin of live bacteria found in breast milk could be the maternal gut and that the bacteria would arrive at the mammary gland through an endogenous route, involving maternal dendritic cells (DCs) and macrophages [145]. Previous evidence suggested that DCs can penetrate the gut epithelium to take up non-pathogenic bacteria directly from the gut lumen [164]. DCs are able to open the tight junctions between intestinal epithelial cells, send dendrites outside the epithelium and directly sample bacteria, while preserving the integrity of the epithelial barrier through the expression of tight-junction proteins [165]. Mechanistic insights revealed that mucosal intestinal DCs regularly engulf intestinal bacteria, which may subsequently be

trafficked into the systemic circulation [164]. Recently, two RCTs compared the effects of *Lactobacilli* strains which are not present in breast milk to antibiotic therapy in mothers who have lactational mastitis. Mothers were randomised to use either lactobacilli or antibiotics for the treatment of lactational mastitis. Results show that oral intake of *Lactobacilli* can be an effective alternative to antibiotics for the treatment of mastitis, reflecting the fact that *Lactobacilli* absorbed in the GI track can be somehow translated to the mammary gland. These findings suggested the existence of a bacterial entero-mammary pathway during human lactation [166, 167]. Taken together, the data suggest that in late pregnant and lactating women, these leukocytes with intracellular bacteria may be trafficked from gut to the mammary gland and secreted into breast milk. Hence, alterations of maternal gut microbiota can potentially alter the microbiota composition in their infant's gut via breastfeeding.

2.5.5. Association between maternal stress, infant behaviour, and the microbiome

As discussed in 1.4.2, there is a bidirectional communication between the CNS and the GI tract, which means that while the gut microflora can alter the host's behaviour, the host's psychological status may also have an influence on modulating the structure of the gut microbiota community. Indeed, animal studies showed that stress can alter the composition of the gut microbiota and this can have marked consequences on psychological development [141, 168, 169]. Baily and Coe showed that maternal separation in rhesus monkeys can increase the mother monkey's stress and thus lead to a substantial decrease in faecal lactobacilli 3 days after the initiation of the separation

procedure, which returned to baseline by day seven[169]. Later studies using 16S RNA sequencing techniques examined the gut microbiota in male mice/rats and demonstrated that stress can alter brain-gut axis function and also modifies the relative diversity of the gut microbiota [170, 171]. Moreover, the changes of gut microflora in mice are also associated with alterations in the stress response and behaviour [131]. Increasing evidence shows that commensal organisms within the gut play a role in early programming and later responsivity of the stress system [131, 172]. Studies in germ-free animals exposed to pathogenic bacterial infections, probiotic bacteria or antibiotic drugs suggest a role for the gut microbiota in the regulation of anxiety, mood, cognition and pain [141, 173]. These findings are consistent with the hypothesis that maternal stress during breastfeeding could influence the mother's gut microbiota, thereby altering the milk microflora, and consequently having an impact on the infant gut microbiota, which could result in changes of infant behaviour (**Figure 2.2**).

2.6. Summary

2.6.1. Limitations of available studies and research gaps

Although there are many interesting and unexplored issues in the signalling between mother and infant during breastfeeding, as discussed above, there are several limitations in previous research studies. Firstly, apart from the MOM study, most previous studies are observational, and cannot identify the complexity of the inter-relationships between factors as well as defining cause and effect, because findings

might also be influenced by various confounding factors. Secondly, existing experimental studies were focused on the effects of intervention on maternal outcomes, rather than infant growth, and mother-infant signalling was also not considered in those studies. Moreover, although the MOM study investigated mother-infant signalling using an experimental design, the sample size was relatively small (n=56), and participants were healthy term infants and their mothers, who are at lower risk for stress and breastfeeding difficulties compared to preterm mothers.

2.6.2. Rationale for proposed study

The present research aims to use an experimental approach, with expanded sample size, to further investigate the causal relationships between maternal psychological state and infant outcomes and the signalling between mother and infant during breastfeeding. In addition, as suggested by the MOM study [174], the potential signalling mechanisms need to be further investigated in larger studies especially in settings where mothers are more stressed. The present research is therefore specifically focussed on mothers of LP and ET infants where there is greater stress/increased tension during lactation. Further, to our knowledge, this will be the first experimental study that investigates the role of microbiota in mother-infant signalling. Findings of this study may provide evidence on gut microbiota and its relationship with behaviour. Results of this study could also identify modifiable factors which can be used to encourage and support exclusive breastfeeding.

Chapter 3

Pilot study

In this chapter, I present the findings of my pilot study which was conducted to identify the most suitable relaxation intervention that could be used in the main RCT. The study used a within-subject design and was carried out from 3rd June to 28th June 2018 in Beijing, China. Results of this study have been published in Breastfeeding Medicine [175] (Appendix 1a).

3.1. Introduction

3.2.1. Rationale and aims

Stress can be one of the factors that influences successful breastfeeding [176]. Increasing evidence has shown that maternal stress can inhibit the milk-ejection reflex; once this inhibition occurs repeatedly, it can lead to lower milk production as a result of incomplete emptying of the breast, consequently affecting breastfeeding duration [108-110]. Moreover, maternal stress was also identified to be associated with elevated serum cortisol level during the postpartum period, which may influence breastfeeding frequency and duration by interfering with the regulation of oxytocin and prolactin [113, 177]. Given this, it has been hypothesised that by reducing maternal stress, the let-down reflex could be stimulated.

The effectiveness of relaxation techniques on reducing stress and anxiety in breastfeeding mothers has been confirmed [14, 19, 118]. Through those studies, a number of relaxation techniques have been tested. Apart from the relaxation meditation that was used in the MOM study, interventions such as relaxation training [178], guided imagery [19], music therapy [179], progressive muscle relaxation (PMR) [125, 126] and a novel relaxation technique, light therapy [180-182] have been tested in a number of experimental studies. Most of the existing intervention studies use the cognitive and/or behavioural relaxation approach which emphasises the development of a relaxation response to counteract the stress response of anxiety [183]. The

relaxation response refers to a set of integrated physiological mechanisms and 'adjustments' that are elicited when a subject engages in a repetitive mental or physical activity and passively ignores distracting thoughts [183]. However, it should be mentioned that the different interventions may also have different effects in people with different cultural backgrounds. Currently, there is a lack of studies evaluating the effectiveness among different relaxation techniques. Therefore, this pilot study aimed to find the most effective and acceptable relaxation technique to help mothers who are breastfeeding their infant in a Chinese population.

The effects of five different relaxation techniques on physical and psychological changes were tested in Chinese mothers who are breastfeeding their first infant. The five interventions were: relaxation meditation tape (RM), music tape (M), relaxation lighting (L), combined relaxation meditation and lighting (RM+L), and combined music and lighting (M+L). The ultimate aim of this study was to select the most appropriate therapies for breastfeeding mothers to be used in a subsequent trial investigating the impact of relaxation therapy on breastfeeding outcomes in mothers who deliver a late preterm or early term infant.

3.2.2. Selection of the interventions

After looking through the relaxation therapies that had been previously tested, three types of the therapy were summarised: 1) guided meditation (verbal relaxation), such as guided-imagery meditation, verbal protocol, and the PMR; 2) music therapy, which

has been defined as “a systematic process of intervention wherein the therapist helps the client to promote health, using music experiences and the relationships that develop through them as dynamic forces of change”[184] and has been frequently used in clinical settings and health professional groups [185, 186]; 3) lighting therapy, which is a new technique that has been used to promote relaxation. Evidence shows that light can stimulate the suprachiasmatic nucleus (SCN) located in the hypothalamus on top of the optic chiasm and influence the secretion of cortisol and adrenocorticotrophic hormone by mediating the HPA axis [187]. Research also indicates that light may induce gene expression ("circadian clock" -related or "sleep"-related genes in depression) in the adrenal gland via the SCN-sympathetic nervous system [188]. An increasing number of studies reported the application of light therapy for the treatment of a range of mental health diseases, namely seasonal affective disorder (SAD) [180], non-seasonal depression [181], total sleep deprivation [182], and antepartum depression [189]. Studies also show that exposure to blue light is associated with reduced heart rate and blood pressure [190], while improved resting metabolic rate was observed in patients with SAD following exposure to bright light [191].

Given this, the relaxation meditation was selected as a representation of the guided meditation. Meanwhile, music therapy and lighting therapy were selected for comparisons. In addition, considering that meditation and music intervention are both based on hearing whilst lighting therapy is based on vision, I also combined the relaxation meditation with lighting as a fourth intervention, and music therapy plus

lighting as a fifth intervention. There was also a control session where participants were asked to sit for 10 minutes in the room with no treatment.

The relaxation meditation used was Sheri Menelli's CD "Breastfeeding meditation" [192] which is specifically designed for breastfeeding mothers and used in the MOM study. The CD contains three parts: 1) introduction, 2) breastfeeding affirmations (9 mins), and 3) breastfeeding meditations (13 mins). In this pilot study, the main concept of the third part was kept, with some breastfeeding affirmations in part two added. The adapted version was 10 minutes long and was translated into Chinese and recorded by a trained yoga master.

The music therapy was tailored for Chinese mothers, so that two types of music could be chosen: new-age music or classical music played by a traditional Chinese instrument; those two types of music are most popular in the "relaxation theme" of a large number of music Apps in China. Each music type contained five most popular songs. Mothers could choose which they liked by trying them before the start of intervention. The duration of each music tape was 10 minutes.

The light therapy used in the pilot study was based on the Philips Hue system, which contains several pre-set modes for different preferences. Two modes were applied in the pilot study: warm-toned orange light ("Relax" setting) and cold-toned blue light ("Energize" setting). Participants could choose either of these to meet their preference and the intensity of the light could also be altered

Table 3.1. Detailed content of the five relaxation therapies that were tested in the pilot study.

Interventions	Coding	Descriptions
Relaxation meditation tape	RM	A breastfeeding meditation tape that is specifically designed for supporting breastfeeding. Guided verbal protocol without any background music.
Music tape	M	New-age music and traditional Chinese music played on a classical instrument. Participants could choose their preferred music.
Relaxation lighting	L	Philips Hue system with two types of lighting provided: orange light ("Relax" setting) and the blue light ("Energize" setting). Participants could choose either according to their preference and the intensity of the light could also be altered
Combined relaxation and lighting	RM+L	Participants listened to the relaxation meditation tape with the Philips Hue lighting
Combined music and lighting	M+L	Participants listened to the music tape with the Philips Hue lighting

3.2. Materials and methods

3.2.1. Study design

The pilot study was conducted at a local community clinic attached to Beijing Children's Hospital in China. A within-subject design was applied where each participant received five different relaxation treatments and one control session with no treatment in random order over three weeks (**Figure 3.1**). The random order of the six sessions was generated using the computer random number generator. Eligibility criteria for inclusion in this study are listed in **Table 3.2**.

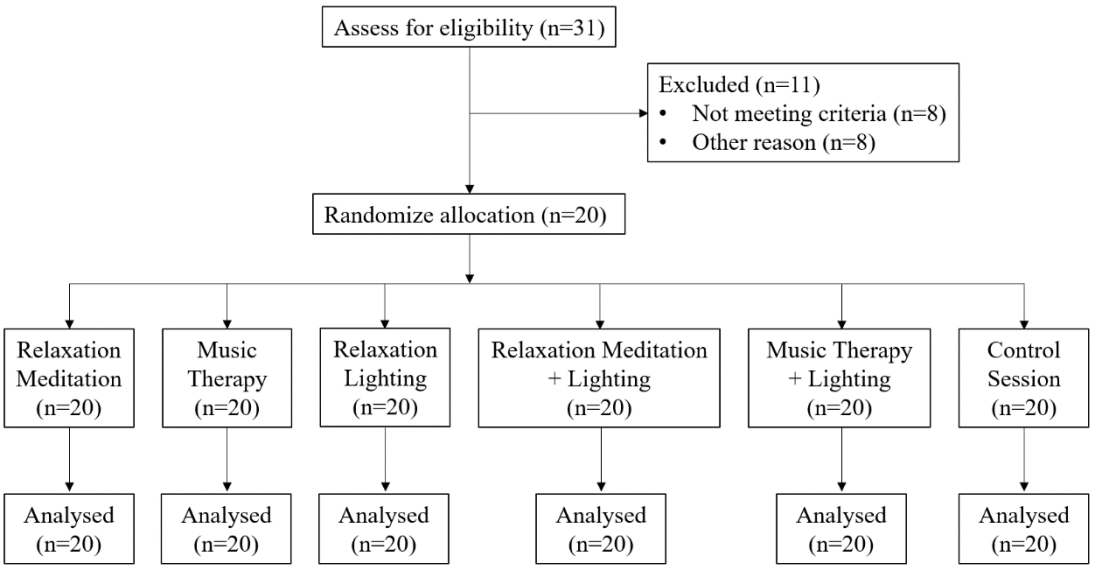


Figure 3.1. Flow chart of the within-subject trial.

Table 3.2. Inclusion criteria for the pilot study.

	Eligibility criteria
1	Primiparous mothers who are currently breastfeeding their infants
2	Age 23 to 45 years
3	Generally healthy (without any diseases that could influence their blood pressure, heart rate, energy expenditure, or breastfeeding practice)
4	Did not attend any other intervention studies within 12 months
5	Non-smoker

3.2.2. Study procedures

The pilot study was conducted from 3rd June to 28th June 2018. Six treatment sessions (five intervention and one control) were coded as 0 = control, 1 =RM, 2 = M, 3 = L, 4 =RM+L, and 5 =M+L. An independent person who had no contact with the subjects generated 20 sequences with 0 to 5 in random order using a computerized random number generator. Each sequence was printed on a card and mothers drew a card at random from the 20 cards without seeing the content of the card. The sequence shown determined the order of treatments for that mother. A washout period around one to three days was arranged between sessions to minimize carry over effects. The order of the six sessions was randomly assigned for each participant. All sessions were performed in the afternoon between 14:00 and 16:00 in order to control for circadian rhythm. The duration of each session was 10 minutes. Moreover, there was a 10-minute pre-test outcome assessment and a post-test assessment before and after each session. All sessions were conducted in a breastfeeding room at the clinic where participants were comfortably seated and guided by the researcher.

Ethical approval for the study was obtained from the Research Ethics Committee of University College London (ID: 12681/001) and the Department of Child Health, Beijing Children's Hospital (ID: 2018-158) (Appendix 2). The trial was registered at clinicaltrials.gov (NCT03593551).

3.2.3. Outcomes and measures

Maternal weight, height, age, and their infant's age were recorded. BMI was calculated based on the formula BMI = Weight (kg) / Height $(m)^2$. Physiological outcomes of the study were changes in heart rate (HR), systolic blood pressure (SPB), diastolic blood pressure (DBP), and fingertip temperature (FT). The SBP, DBP and HR were measured in the seated position using an electronic blood pressure machine (Yuyue-660C, China) by trained nurses with at least 10-minute rest period before the pre-treatment measurement. The post-treatment measurement of blood pressure was conducted immediately after the treatment. The fingertip temperature was measured using a digital body thermometer (Care1st-DT8836, China). All assessments were carried out three times and the mean of the closest two readings was recorded.

The psychological outcome of the study was maternal perceived relaxation, assessed using a visual analogue scale (VAS). The VAS can show subjective experience of immediate short-time effects of the intervention and has shown good within-subject reliability and validity [193]. The VAS consisted of a horizontal 10 cm line. The left anchor of the line was labelled "completely unrelaxed" while the right end represented

"completely relaxed". Mothers were asked to mark their state of relaxation on the line before each session and again at the end of each session. The distance from the left anchor to the mark made by participants was measured in millimetres. Pre- and post-test differences were compared.

3.2.4. Sample size calculation

To estimate the sample size for this pilot study, the standard formula [181] used for calculation is as follows:

$$Sample\ size\ (per\ equal-sized\ group) = \frac{8 \times standard\ deviation}{difference^2}$$

Here the effect size and standard deviation (SD) are estimated from a previous research study[194], which evaluated the effect of audio-visual imagery on patient anxiety and physiological parameters; and a significant reduction in HR was observed (mean change (Z)= -0.75, SD= 1.00; p=0.01). Using these figures in the formula, a sample of 14 participants would be required. Considering the potential for drop-outs, I planned to involve a total of 15-20 subjects in this study.

3.2.5. Statistical analysis

Statistical analysis was conducted using SPSS 26.0. The distribution of the data was examined by histogram. Box-plots were used to provide references for the exclusion of outliers. Descriptive analysis was used to calculate mean and standard deviations (SD)

of participants' characteristics. Means and SD were used to summarise the characteristic of the entire sample. Paired t-tests were carried out to detect changes in each of the outcomes before and after the sessions. Paired t-tests were also computed to compare the mean difference of each intervention to the control state. Differences were considered statistically significant at $p<0.05$.

3.3. Results

A total of 20 primiparous mothers participated in the study, 16 of them were either currently exclusively breastfeeding or had breastfed their infant for at least 5 months after birth. The age of the participating mothers was 32.2±3.3 years (range 28-38 years), and the age of their infants was 7.55±6.18 months (range 1-24 months). **Table 3.3** outlines the characteristics of the mothers and their infants. There were no significant differences between participants for maternal height, weight, and BMI. No participants were unwell during the study period.

Table 3.3. Characteristics of the study population.

Characteristics	N (%)/Mean (SD)
Maternal age (years)	
23-30	6 (30)
31-35	10 (50)
36-40	4 (20)
Maternal weight (kg)	58.5 (4.5)
Maternal height (cm)	162 (3.3)
Maternal BMI	22.4 (1.8)
Infant age	
0-6 months	11 (55)
7-12 months	7 (35)
13-18 months	0 (0)
18-24 months	2 (10)

Notes: SD= standard deviations.

As shown in **Figure 3.2**, all five treatments showed reduced blood pressure (BP) and HR and increased FT and perceived relaxation after the session, whilst in the control session, the pre-post changes in SBP, HR and perceived relaxation were very small and the DBP and FT changes were opposite to those in the intervention groups. Specifically, the relaxation meditation, meditation plus light, and music plus light groups show significant pre-post changes for all five assessed outcomes (SBP, DBP, HR, FT, and perceived stress,

all p<0.05). For music therapy, significant pre-post changes were found in DBP (t=2.289, p<0.05), HR (t=3.714, p<0.01), FT (t=-2.737, p<0.05), and perceived relaxation (t=-7.175, p<0.01) but not in SBP (t=1.497, p>0.05). Moreover, results show that for all six sessions, the perceived relaxation is significantly increased after the treatment (p<0.01). The pre- and post-test mean values of each outcome and the values of changes are shown in Appendix 1b.

Figure 3.2. Pre-post changes among six sessions
Notes: VAS= visual analogue scale. *Significant pre-post changes.

Comparisons with regard to the changes in all five assessed outcomes among the six sessions (five treatments and the control session) were conducted using repeated paired t-test. The relaxation meditation showed significantly reduced SBP, DBP, HR and significantly increased FT and perceived relaxation compared to the control session. Moreover, all treatment sessions presented significantly higher FT and perceived relaxation compared to the control state ($p<0.01$). Compared to the control session, the meditation + light and music + light session showed significant reduction in DBP, but not in SBP.

Further, it was notable that four participants (number 6, 7,11,12) reported having a let-down-reflex (milk ejection during the test) when they were testing the RM treatment. The age range for those participants was 28-38 years while the age range of their infants was 1-3 months. No other reports about the let-down-reflex were found in the remaining four treatments or control state.

3.4. Discussion

This study found that the five-relaxation interventions tested had a positive impact in terms of perceived relaxation and fingertip temperature in comparison to the control state in a sample of Chinese mothers that were breastfeeding their infants. Compared to the pre-test state, there was a significant decrease in blood pressure and heart rate following the RM, RM+RL, M+RL sessions; in addition, all three treatments produced significant improvement in FT and perceived relaxation post-test. Comparing the pre-

post test changes for the relaxation interventions with the control state, the RM resulted in the highest mean difference on SBP, DBP and HR while the M+L showed the highest mean difference in FT and perceived relaxation. Moreover, when compared to the control state, the RM presented significant pre-post test changes in all measured outcomes (BP, HR, FT, and perceived relaxation) in comparison to the control state. All 20 participants attended their six sessions during the study period, reflecting a high level of engagement and sustained involvement considering they were asked to attend six times during the study.

Studies in other populations had similar findings. A randomised controlled trial (RCT) compared the effects of music, progressive muscle relaxation, music assisted progressive muscle relaxation, and silence on measures of anxiety and perceived relaxation in 60 university students with an average age of 22.2 years [195]. VAS and State Trait Anxiety Inventory (STAI) were used to measure perceived relaxation and anxiety. All participants showed significant relaxation with all the treatments. However, this study did not include any assessment of physiological responses. Participants reported their relaxation based on a self-rating scale; this can lead to reporting bias as the participant may expect to be relaxed after treatment.

To minimise the aforementioned bias, my study used three physiological outcomes together with VAS for the evaluation of relaxation. VAS outcomes increased following each session as shown in Figure 3.2, including the control session. There were no significant changes in other physiological outcomes during the control session.

Therefore, it is questionable if VAS is a reliable indicator of the participant's response to the interventions.

As suggested by Gay Peterson [196], the stress response is complex and typically encompasses elements of cognition, physiology and behaviour. Therefore, depending on the individual's response to stress, different relaxation techniques might be more appropriate or effective for different individuals. Physiological and cognitive stress can be reliably detected through physiological and perceived indicators respectively. It is possible that people may experience a similar level of cognitive relaxation using different relaxation techniques or just sitting quietly; however, significant cognitive relaxation effects which can be detected by perceived indicators may not be identified by physiological indicators. However, there is limited evidence on this topic and it is hard to know whether perceived or physiological relaxation is most relevant in terms of improving breastfeeding. More experimental studies in breastfeeding mothers are recommended.

Another previous study employed a RCT to analyse perceived and physiological signs of stress in 56 undergraduate students with an average age of 21 years by exposing them to various types of music and silence [197]. Assessment was made of both perceived and physiological indicators. Physiological indicators included frontalis muscle tension, heart rate and skin temperature. Meanwhile, the perceived indicator was a relaxation rating scored by participants using a scale. Results showed a significant increase in perceived relaxation ($p = 0.004$) when the participant listened to classical music, self-selected

relaxing music and no music. However, the same participants did not have significant changes in terms of their physiological indicators. Again, this suggests that while these participants may subjectively feel they were relaxed after listening to certain types of music, they were not physiologically "relaxed". In other words, they might expect themselves to be "relaxed" after the treatment.

Apart from perceived relaxation, RM was shown to produce the greatest effect on SBP, HR and DBP. Moreover, four participants reported having a let-down-reflex exclusively during the RM session. As suggested by previous research, the let-down-reflex is affected by maternal psychological state, so milk ejection could be stimulated by reducing the maternal psychological distress [18, 198]. Although this was an anecdotal finding in four women, it suggests that the RM may have the greatest effects among all five treatments on milk ejection, potentially by stimulating the let-down-reflex. Hence, the RM might be particularly suitable for use in breastfeeding women.

This pilot study is the first experimental study that examined the acute effects of five relaxation techniques on reducing physiological and perceived stress in breastfeeding mothers. The use of a within-subject design allowed several relaxation interventions to be tested and compared in a single study. The pilot study also had a high completion rate. However, there are some limitations that should be acknowledged. First, all participants were from one community in Beijing which limits generalisability. Second, the population in the pilot study were term mothers, whilst the target population of the planned RCT is mothers of LP and ET infants. However, as a pilot study and with limited

time available it would have been difficult to recruit a larger sample from multiple clinics across Beijing, especially if recruitment had focussed on mothers of LP and ET mothers. Considering the aim of this pilot study was to discover the most suitable relaxation technique for a planned RCT in breastfeeding mothers in Beijing, the sample was reasonably representative with respect to the geographic location and inclusion of breastfeeding mothers. Third, the sample size was relatively small. However, the within-subject design limited variability and required a smaller sample size to detect a given effect size than would have been the case for a between-subject study.

The fourth limitation is time-related effects and "carry-over effects" due to the study's within-subject design that might bias the results. However, to minimise bias, the order of the six sessions assigned to each participant was randomly assigned thus the participants did not have prior expectations about the outcome of each session. Besides, the carryover effect was also minimised by having a washout period of 1 to 3 days between each session.

3.5. Conclusion

Findings of this pilot study suggested that several different relaxation techniques can reduce physiological and perceived stress in mothers who are breastfeeding their infants, but the RM may be the most effective technique among the five tested treatments and control state. Therefore, the RM was selected as the intervention for the planned RCT. The next chapter describes the methodology for the RCT, which aimed to investigate the

effects of RM on maternal psychological status, infant growth, and breastmilk composition following LP and ET delivery.

Chapter 4

Methodology of a randomised controlled trial investigating the effects of relaxation therapy on maternal psychological status, infant growth, and breastmilk composition in mothers of late preterm and early term infants

Based on the findings of the pilot study described in chapter 3, the relaxation meditation tape performed the best for reducing maternal stress compared to other relaxation interventions and was chosen to be the intervention in the main RCT. The aim of this RCT was to assess the effects of the relaxation meditation tape on maternal psychological changes and infant growth after longer-term use (from 1- week to 8- week postpartum). The protocol for this RCT has been published and can be found in Appendix 3 [199]. In the following sections, I provide details of the methods, procedures, outcomes and assessments, and also the statistical analysis of the main RCT.

4.1. Overview of the study

As discussed in previous chapters, the target population for this RCT was mothers of late preterm and early term infants. A single blind parallel RCT was conducted in primiparous mothers who delivered at 34 0/7–37 6/7 weeks and planned to exclusively breastfeed. Screening for eligible participants was conducted using the database from local clinics located in four districts of Beijing, China. Eligible mothers were contacted 3-5 days after birth. After obtaining written informed consent, participants were randomly assigned to either intervention (listening to relaxation meditation) or control group (no treatment, normal care) (IG, CG) using assignments held in sealed opaque envelopes. Mothers who were allocated into the IG were asked to listen to a relaxation meditation audio recording daily during breastfeeding while CG mothers received no intervention. Both groups received standard breastfeeding support and postnatal care. Two home visits were arranged around 1-week and 8-weeks postpartum. Demographic information, infant feeding attitudes, infant eating behaviour, maternal stress and anxiety were assessed using standard questionnaires (Section 4.6). Infant weight and length were measured using standard anthropometry method during home visits.

4.2. Research hypothesis and outcome measures

4.2.1. Primary hypotheses

The use of relaxation therapy by breastfeeding mothers following late preterm and early term delivery starting at 1-week postpartum, will result in:

i. reduced maternal stress and anxiety

ii. increased weight and length gain compared to control group.

4.2.2. Secondary hypotheses

The use of relaxation therapy by breastfeeding mothers following late preterm and early term delivery will result in

1) increased total carbohydrate and HMO in breast milk at the endpoint (8 weeks postpartum);
2) increased breast milk volume and energy at 8 weeks postpartum;
3) differences between IG and CG regarding the microbial diversity in maternal gut and breastmilk, as well as in the infant's gut at 8-weeks;
4) differences between IG and CG regarding the changes of microbial community structures in maternal breast milk, gut, and the infant's gut from 1- to 8-weeks;

4.3. Outcome measures

4.3.1. Primary outcomes and measures

1) changes in maternal stress and anxiety from baseline to 8 weeks. Maternal stress and anxiety were assessed using the 14-item Chinese version of Cohen's Perceived Stress Scale (PSS-14) and Beck Anxiety Inventory (BAI) respectively.
2) infant weight and length gain (as SD scores) from 1 to 8 weeks postpartum measured using anthropometry.

4.3.2. Secondary outcomes and measures

1) composition of macronutrients: fat, protein, carbohydrate in breast milk.
2) breast milk intake at 8 weeks assessed using the 48-hour test-weighing method.
3) breast milk energy content: total energy content was obtained from the results provided by the milk analyser (calculated by using conversion quotients of 9.3 kcal/g for fat, 4.0 kcal/g for carbohydrate and protein) [200]
4) composition of breast milk microbiota and maternal and infant's gut microbiota, using the 16S rRNA based amplicon sequencing technique (details shown in Chapter 7).
5) infant appetite assessed using the Baby Eating Behaviour Questionnaire (BEBQ) at 8-weeks [201], maternal breastfeeding attitudes assessed using the Iowa Infant Feeding Attitude Scale (IIFAS) [202].
6) infant behaviour measured by 3-day infant behaviour diary [203, 204].

4.4. Study framework

4.4.1. Study design

- Single-blinded parallel randomised controlled trial

4.4.2. Research setting

Four study centres in different Districts of Beijing were selected for the recruitment (**Figure 4.1**), including northeast Beijing (Shunyi District, Centre A), central Beijing (Haidian District, Centre B), northwest Beijing (Changping District, Centre C), and south Beijing (Daxing District, Centre D)

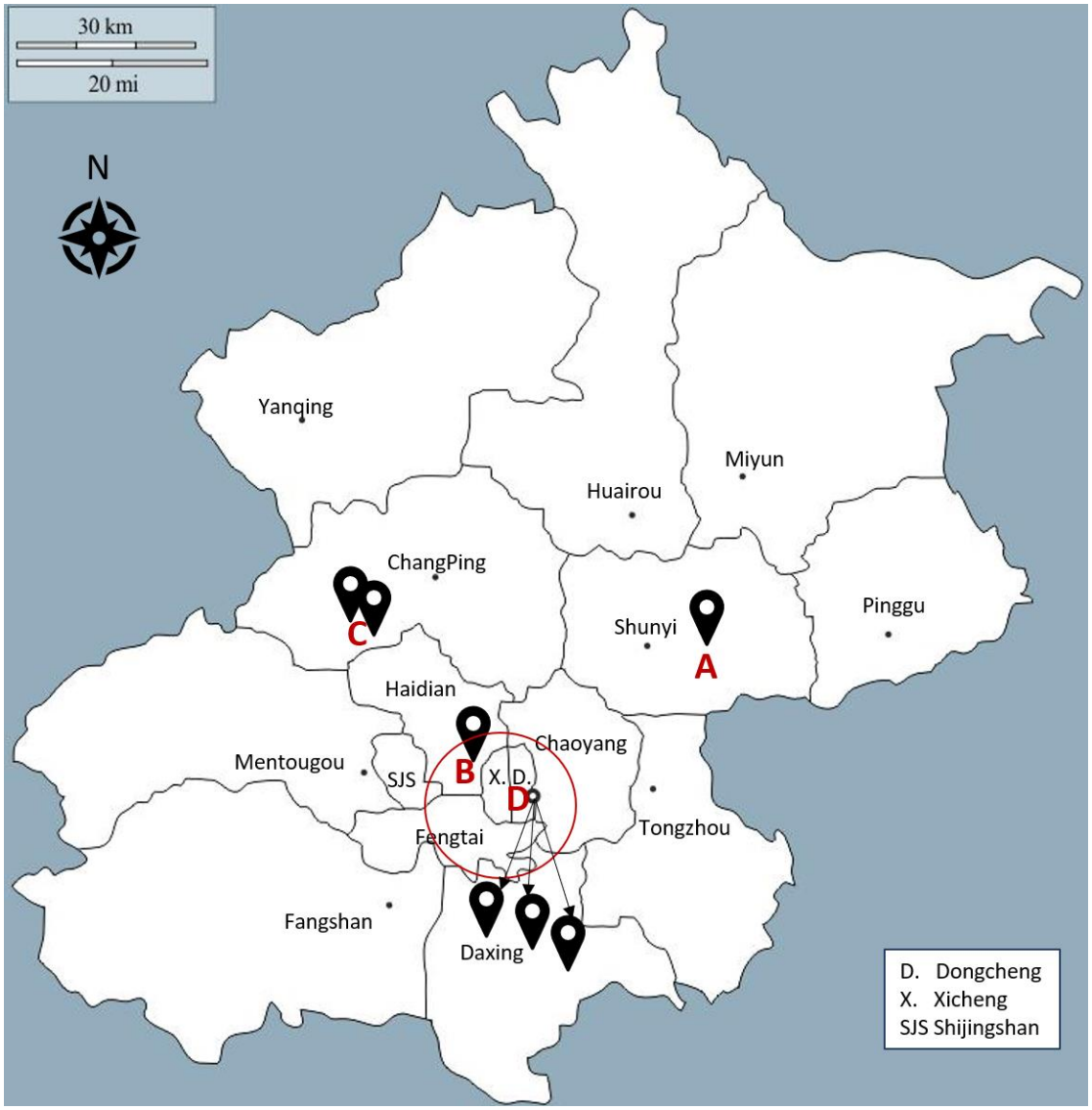


Figure 4.1. Centres for study recruitment and data collection: the map of Beijing by districts

Notes: The circled area represents the Five Ring Road of Beijing, which is generally acknowledged as "central Beijing".
Study centre A: Shunyi Maternal and Child Health Hospital
Study centre B: Haidian Maternal and Child Health Hospital
Study centre C: Huilongguan Community Health Centre (including two clinics)
Study centre D: Bayi Children's Hospital (All participants from this centre lived in Daxing District, where the actual data collection was conducted).

4.4.3. Participant recruitment

The study population were recruited from local community clinics located in four districts of Beijing. Parents of infants who were eligible were screened 2-3 days after delivery when they were in the hospital and breastfeeding was successfully established. Research assistants (research students and nurses at the BCH) approached parents and briefly introduced the content of the study and the measurements which would be undertaken. The information sheet including the study details was left for the mother to consider. To ensure the study procedures were consistent at each study centre, all research assistants and nurses that were involved in the study attended training courses prior to the start of recruitment. Standard operating procedures for the study were printed and posted at each study centre. Detailed information for staff training is shown in next section.

Table 4.1 outlines the eligibility for inclusion to the RCT. After discharge from the hospital, eligible mothers were contacted by the local clinic to see if they were interested in taking part. According to the health policy of Beijing, a regular postpartum home visit was conducted around 1-week (5-12 days) postpartum for each new mother. Nurses from local clinics provide advice to the mother and take infant weight and length measurements during the home visit. Therefore, the first home visit of the BABY study was scheduled together with the regular home visit at each local clinic. Nurses introduced the BABY study again to the mother and the consent form was signed if the mother wanted to participate.

Table 4.1. Eligibility criteria for inclusion in the RCT.

Inclusion criteria	Exclusion criteria
Primiparous mothers	Multiparous mothers
EBF mother who is aiming to exclusively breastfeeding the infant for at least two months [a]	Mothers who did not intend to EBF or could not EBF due to health conditions.
Infant is a singleton born late preterm or early term (34 0/7–37 6/7 weeks of gestation)	Infant who was not singleton born or infants born before 34 0/7 weeks or after 37 6/7 weeks
Mother and infant are generally healthy (free of serious diseases that can affect breastfeeding or the growth of the infant)	Infant with serious underlying or chronic disease [b] Infant receiving any medication regularly, apart from vitamins.
Non-smoker	Mothers who smoke

Notes: [a] Considering that breastfeeding in LPI or ETI mothers might not be established immediately after delivery; it was acceptable if the baby had received some formula or expressed breastmilk initially. However eligible participants had to be EBF at enrolment. [b] For healthy infants who had an acute illness, the visit was re-scheduled within 1-2 weeks.

4.4.4. Study procedures

1) Randomisation and blinding

Randomisation was conducted after obtaining written consent from the participants. To ensure that the number and characteristics of subjects assigned to each group was equally distributed, randomisation was stratified by gestational age, delivery method (34-35 weeks vaginal, 34-35 weeks caesarean, 36-37 weeks vaginal, 36-37 weeks caesarean) and by the study centre (located at four districts in Beijing). Then, subjects within each block were randomly assigned to either intervention or control groups (IG, CG). IG mothers were asked to listen to a relaxation meditation audio recording daily during breastfeeding; CG mothers received no intervention. Both groups received standard postnatal care and breastfeeding support. For the allocation concealment, an independent investigator, Professor Fewtrell, generated the study ID (randomisation sequence) using a computer random number generator. The assignments were stored in sealed, opaque envelopes and sent to the research team at the BCH. Considering the multicentre nature of the trial we used a remote randomisation facility, using the Chinese message App "Wechat". All research assistants and nurses involved in study recruitment were members of the group. When there was an eligible participant who wanted to take part in the study, the nurse typed the details (eg. "36 weeks vaginal centre A" or "35 weeks caesarean centre C") in the chat group, and an independent research assistant at the BCH opened the relevant envelope and sent the study ID and the randomisation assignment for the subject.

Participants were blinded to the randomisation until the end of the study; they were aware that the aim of the study was to investigate factors that may make breastfeeding easier for new mothers. Due to the nature of study, the researchers could not be blinded since additional materials (diary for recording the use of relaxation tape) were collected during the data collection period.

2) Staff training

To ensure the study procedures could be standardised, formal meeting and seminars were arranged prior to the start of the recruitment. All research assistants and nurses attended the simulation training, including the method for remote randomisation, the use and explanation of all study questionnaires, infant anthropometry measurement, and the methods for collecting biological samples. Questions regarding the study procedure were recorded and solved during the seminar. Standard operating procedures for study recruitment and data collection during home visits were posted in the office at each study centre. All data were double checked before being entered into the computer database; mothers were contacted by local clinical nurses to clarify, where possible, any odd results observed from their questionnaires during the analyses.

3) Home visits and data collection

Home visits were arranged at 1-week (5-12 days) and 8-weeks (50-62 days) postpartum in the morning. Maternal stress, anxiety, infant behaviour and appetite were measured

using standard questionnaires at each home visit; participants could choose to complete the questionnaires during the home visit or in their own time after the visit. A breast milk sample was collected pre-feed and infant anthropometry was assessed by a trained nurse pre-feed at each home visit. Feed duration was noted by the trained nurse. Stool samples of infants who were born vaginally were collected by mothers at baseline and the final home visit, with detailed instructions provided by the nurse. Instructions for milk and stool sample collection are shown in Section 4.4.6. Details about other data collection at each visit are as follows:

First visit at 1-week postpartum:

- Demographic questionnaire: Family type, family income
  - Maternal age, education, occupation, height, BMI
  - Prenatal problems, mode of delivery, gestational age at birth
  - Infant gender, birth weight, post-natal problems
  - Types of infant feeding in the first 2 weeks, any therapies needed during postnatal confinement (mother and infant)
  - Infant health
- Anthropometric data: Maternal and infants' weight and height (length for infant)
- Maternal stress and anxiety: Chinese version of PSS and BAI
- Infant appetite: Chinese version of BEBQ

- Infant questionnaires: The Chinese version of Iowa Infant Feeding Attitudes Scale (IIFAS); 3-day Infant Behaviour Diary
- Biological samples: breast milk samples and infant stool samples
- Other information: Infant health (including feeding problems)

Second visit (final visit) at 8-week postpartum:

- Anthropometric data: Maternal and infants' weight and height (length for infant)
- Maternal stress and anxiety: Chinese version of PSS and BAI
- Infant appetite: Chinese version of BEBQ
- Infant questionnaires: Chinese version of IIFAS; 3-day Infant Behaviour Diary
- Milk intake: test-weighing method for the assessment of milk intake
- Biological samples: breast milk samples and infant stool samples
- Other information: diary about the frequency of using the relaxation tape (for intervention group mothers), feeding problems, acute illness and antibiotic use since last visit.

4.4.5. Intervention and control

Intervention group mothers were asked to use the relaxation therapy during a breast-feed at least once a day. The purpose of the relaxation exercises was explained to the mother at the first visit. Mothers were given a diary to record when it was used.

Following the baseline measurement, mothers in the intervention group were asked to continue with their intervention as often as they found it helpful.

The tape used in this study was based on a meditation CD designed for breastfeeding mothers [192]. The CD consisted of three parts: i) introduction and instruction for the CD; ii) breastfeeding support, which could help to improve mother's attitudes towards breastfeeding; iii) breastfeeding meditation, which could help to relax the mother's mind and body. The recording was transcribed and translated into Chinese language by a certified yoga therapist. A brief version of the recording was tested and compared with other four relaxation techniques in the pilot study (Chapter 3) and was demonstrated to be the most effective approach for breastfeeding mothers to relax [205]. Mothers in the intervention group were given the tape by scanning a QR code. They were asked to listen to the recording as frequently as possible while breastfeeding or expressing milk, preferably at least once a day. They were also asked to record their use of the tape in a diary book. Routine care and postpartum support were arranged as usual for mothers in both groups. Mothers in the control group were not told anything about the relaxation therapy.

Control group mothers received normal care as usual; aa home visit was arranged around 1-week after birth, anthropometry measurements for mother and infants were conducted and advice on breastfeeding and infant care were provided. In clinical settings in Beijing, mothers of preterm infants were recorded by their registered local clinics. However, compared to moderate and very preterm delivered mothers who

receive special care and additional telephone interviews during and after the home visit, mothers of healthy LP infants receive similar care to term mothers. The primary reason is that the nurses do not want to make LP mothers feel that their infant is "vulnerable". Instead, if the infants are generally healthy, nurses will encourage mothers that they are able to successfully EBF in the same way as term mothers. Mothers were also encouraged to contact their registered personal doctor when experiencing any issue on infant feeding or with their own health personal.

Both IG and CG mothers received additional breastfeeding support from myself, the paediatric consultant, or Dr. Wei (the paediatrician at BCH) via Wechat. They were able to create an individual chat with us if they faced breastfeeding problems. The frequency of individual chats was recorded and the number of episodes for each participant are shown in Appendix 6; there was no significant difference between IG and CG.

4.4.6. Study materials and sample collection

1) Breast milk sample collection

Maternal foremilk samples were collected for the analyses of milk composition and microbiota. The collection was conducted at 1-week and 8-week home visit using a hand pump (Philips Avent, Netherlands), which was provided to the mother as a gift for participating in the study. Prior to

collection, mothers were asked to clean their nipples and areola with soap and sterile water to reduce contamination by skin flora. The first drops of milk (approximately 500 μL) were discarded. Mothers were instructed by a trained nurse on how to express milk using the pump. The nurse collected 20ml foremilk and poured the milk into four sterile specimen jars (5ml per jar) (**Figure 4.2**). Samples were frozen immediately in a cooler box and transported to the -80°C refrigerator in the laboratory (Lab) of the BCH. Samples for future microbiota analysis were collected by a biomedicine company (Novogene Technology Inc., China) using professional cold chain transportation after the completion of the data collection. Breast milk macronutrient analyses were conducted in the BCH laboratory by myself during the study period.

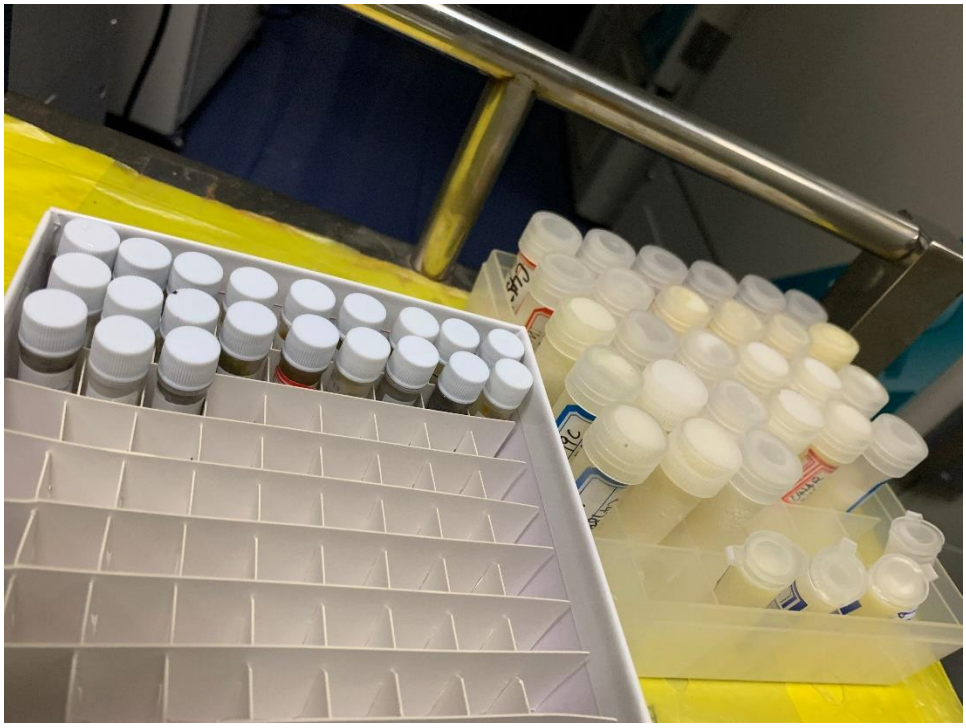


Figure 4.2. Breast milk samples and stool samples

2) Stool sample collection

Infant stool samples were collected by the trained nurse and kept in a white capped opaque specimen jar. To collect the sample, the nurse laid the nappy out flat, opened the specimen jar, and using the scoop built into the lid of the specimen jar collected a small amount of faeces (about the size of a soybean) from the nappy. The nurse placed the scooped stool sample into the specimen jar and firmly screwed the lid on the specimen container. Each jar was labelled with the study ID, date and time the sample was collected. The sample was frozen immediately in the cooler box transported to the -80°C refrigerator in the Lab of the BCH to keep the microbiome stable. When the mother or infants did not have a sample ready for collection at the time of

the home visit, a clear instruction for sample collection was left for the mother, along with the disposable gloves, specimen jar, and cooler bag (insulation bag with dry ice). The mother could collect the sample herself when prepared. Once the sample was collected, the mother was asked to put it in the cooler bag then send to the local clinic within 24 hours.

3) Milk composition

The content of different substances in breast milk can be tested according to the different light transmittance of components in breast milk (fat, carbohydrate, protein, etc.) to the light of short-wave band, medium wave band and long wave in the near infrared spectrum. The macronutrient content of breast milk in the study was hence measured using a Mid-infrared milk analyser (HLIFE, China) at the Lab of BCH (**Figure 4.3**), including the content of fat, total carbohydrate content (both lactose and oligosaccharide), protein (without non-protein nitrogen and crude protein), and total milk energy (kcal/100ml) of the breastmilk. The milk sample is sucked into a customized quartz cuvette with 1 mm thick walls by a peristaltic pump, then, in the quartz cuvettes, the milk sample is irradiated with four different wavelengths of light (the light source is customized to measure fat, carbohydrate and protein content specifically, based on the functional groups for each macronutrient in human breast milk through waveband filters). Using a spectrophotometer, the transmittance milk spectra with

different wavelengths were obtained from analysed samples and were recorded in the linked computer as absorbance. The content of each macronutrient can then be identified based on the published value of absorbance for fat, protein, and carbohydrate. Prior to spectral analysis, each sample was thawed at room temperature (27-29°C). The analyser was set up in calibration mode for homogenised human milk based on the manufacturer's guideline. Studies on the NIRS technique for composition of human milk are well-established and have shown good correlation with reference values [206-208]. The analyser I used was accredited by the China Medical Equipment Association as suitable for use by medical institutions at all levels. The error of detecting the same milk sample is ≤ 0.1% compared to the national standard code for Chinese characters (GB / T5413.3-2010).

Figure 4.3. Analysis of the macronutrient composition in breast milk.

4) Breast milk intake (48-hour test weighting)

A digital electronic infant weight scale (Hochoice, Shanghai, China) accurate to 1g was lent to the mother for 48 hours at the 8-week home visit. The timing and duration of each feed were recorded by the mother. Mothers were informed to take care and ensure that the infant was weighed with the same clothing before and after the feed. The difference in weight was the amount of breastmilk the infant consumed during the feeding. The 24-hour milk intake was estimated by i) calculating the volume of milk intake from each feed by subtracting the pre-feeding infant weight from the post feeding infant weight; ii) summing the volumes of each feed from the beginning of the first feed of the first day to the last feed on the last day; ii) dividing by the total feeding

frequencies; iii) multiplying by average feeding frequency per 24-hours. Moreover, in order to correct for insensible water losses (IWL) during feeding, which is approximately 5% based on the published literature [209], the final value was obtained after adding 5% of the calculated value.

5) Breast milk energy content calculation

Breast milk total energy content was obtained from the results provided by the milk analyser (HLIFE, China). As suggested by the manufacturer, the gross energy of breast milk was calculated by using following equation: Energy (kcal/100ml) = (9.3 kcal/g * fat g/100ml) + (4.0 kcal/ g * protein g/100ml) + (4.0 kcal/g * carbohydrate g/100ml).

6) 16S rRNA-- Sequence based Gut Microbiome Profiling

The composition of microbiota in breast milk and faecal samples was examined using the 16S ribosomal RNA (rRNA) based amplicon sequencing technique. 16S rRNA is located on the small ribosomal subunit of prokaryotic cells, including 10 conserved regions and 9 hypervariable regions. Among them, the conserved regions have little difference among bacteria, and the hypervariable regions have genus or species specificity, which vary with different genetic relationships. Therefore, 16S rRNA can be used as a specific nucleic acid sequence to reveal biological species, and is considered to be the most suitable index for bacterial phylogeny and classification.

Polymerase chain reaction (PCR) is a technique that uses a piece of DNA as a template to amplify the DNA to a sufficient number with the participation of DNA polymerase and nucleotide substrate for structural and functional analysis. 16S rRNA genes in fecal and breast milk bacterial DNA were amplified by universal primers, sequenced by Illumina HiSeq technology (NovaSeq), and aligned for taxonomic classification to microbial genomes using the QIIME pipeline. By detecting the sequence variation and abundance of the target region, the species classification, abundance, population structure, phylogeny and community comparison of environmental samples can be analysed. Detailed explanation of the microbiota analyses is provided in Chapter 7.

7) Anthropometric assessment

Measurements on mothers

Mothers were weighed at the 1-week and 8-week home visit. Mothers were asked to empty their bladder and then weighed in minimal clothing to the nearest 0.1 kg using an electronic scale. The measurement was repeated three times and the mean value was recorded.

Measurements on infants

Infant birth weight was recorded from the birth certificate or from parental recall. At 1- and 8-week home visits, the anthropometry assessment was

conducted following the detailed instructions provided by WHO (available at: http://www.who.int/childgrowth/training/en/index.html). Recumbent length and weight of infants were measured using the electronic infant weight and length scale (Betterren-FSG-25-YE, Shanghai, China) (**Figure 4.4**). Each measure was repeated three times and the mean value used. Weight-for-age and length-for-age z-scores were calculated using 21st intergrowth reference data and WHO term infant reference data.

Figure 4.4. Scale used for measuring infant weight and length.

Infant length was measured to the nearest 0.1 cm on the recumbent board of the infant scale. Two trained nurses measured the infant's length. One person assisted in positioning the child face up on the measuring board, supporting the head and placing it against the headboard. The infant was placed lying straight along the centre line of the board; with shoulders touching the board, and the spine flat. The second person placed one hand on the shins above the ankles or on the knees and pressed down firmly. The foot piece was placed firmly against the heels with the other hand.

The infant was weighed without clothes using the electronic weighing scale. A towel was placed over the scale before resetting to zero. Infant weight was recorded to an accuracy of 0.001 kg.

8) Maternal questionnaires (whole questionnaires are listed in Appendix 4)

a) Cohen's Perceived Stress Scale (PSS)

PSS is a psychological self-rating scale for measuring the perception of stress on a scale of five, from 0 (never) to 4 (very often). The original 14-item English version of PSS was developed by Cohen and his colleagues. As a global measure of perceived stress, it appears to be reliable and validated for the measurement of stress in chronic conditions. The present study used the translated Chinese version of PSS, which has been validated for the Chinese population (Cronbach's α=0.86) [210].

b) Beck Anxiety Inventory (BAI)

BAI is a 21-question multiple-choice self-reported inventory that is used to measure the severity of anxiety in psychiatric populations. Measures are obtained on different symptoms of anxiety on a scale of four, from 0 (not at all) to 3 (severe). Research demonstrated that the BAI has high internal consistency and reliability (Cronbach's α=0.94). The present study used the translated Chinese version of BAI, which has been validated for the Chinese population (Cronbach's α=0.95) [211].

c) Baby Eating Behaviour Questionnaire (BEBQ)

BEBQ is derived from an existing psychometric measure validated for older ages, the Children's Eating Behaviour Questionnaire, supplemented by a review of the literature on milk-feeding behaviours. It has been used in a large birth cohort study in the UK (n=4804), and appears to be reliable with Cronbach's alpha values ranging from 0.73 to 0.81[201]. BEBQ can be used to measure infant appetite and eating behaviour during the period of exclusive milk feeding, which makes it well-suited for neonates. It consists of 18 items designed to measure four traits: "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 all items based on a scale from 1 (never) to 5 (always).

d) The Iowa Infant Feeding Attitude Scale (IIFAS)

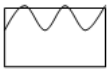
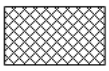
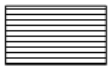
The questionnaire is designed to measure maternal attitudes toward infant feeding methods. It consists of 17 questions and the mother was asked to give her opinion based on a scale from 1 (strongly disagree) to 5 (strongly agree). This Chinese version of IIFAS has been used extensively and has been tested for reliability with Cronbach's alpha 0.74 [202].

e) 3-day Infant Behaviour Diary

Infant crying behaviour was recorded at 1- and 8-weeks home visit using a validated 3-day diary. The diary consists of a "time ruler" for 72 hours, which is divided into 15 minutes segments, and has five categories of behaviour:

Sleeping, Awake and content, Fussy, Crying, Colic, and Feeding [203, 204]. Mothers were asked to shade on the 'time ruler' using the appropriate symbol for the infant behaviour. The length of shading represented how long the behaviour lasted for. The definition of each behaviour and its symbol on the dairy are shown in **Table 4.2**. The original behaviour dairy can be found in the Appendix 4.

Table 4.2. Definitions and symbols of the infant behaviour on 3-day infant behaviour dairy.

Infant behaviours	Symbol	Definition
Sleeping		The time that infant is asleep
Feeding		The time when feeding the infant
Awake and happy		The time that the infant is awake and happy (content)
Crying		periods of prolonged distressed vocalisation
Fussing		baby is unsettled and irritable and may be vocalising but not continuously crying
Colic		bouts of intense, unshootable crying and other behaviour, perhaps due to stomach or bowel pain

Notes: The definition of symbols and instruction of use of the 3-day infant behaviour dairy are presented in the questionnaire, which can be found in Appendix 4.

4.4.7. Monitoring of compliance

To encourage compliance with exclusive breastfeeding, mothers were contacted by telephone once a week from the local clinics during the 1- to 8-week period postpartum. Breastfeeding support and advice were provided for all mothers regardless of randomisation. I also provided my personal contact number to all mothers and they were encouraged to ask any question related to breastfeeding.

4.5. Statistical considerations and analysis

4.5.1. Sample size calculation

The number of mother-infant pairs required was calculated using the conventional formula [212] for a two-sample t-test:

$$N = 16 \times \frac{SD^2}{D^2}$$

(N=number per group, SD=standard deviation, D=Difference between group)

The SD and D were obtained from the results of the MOM study, which assessed the effects of relaxation meditation tape on reducing maternal stress assessed by PSS between intervention and control groups (D=3.13, SD=5.00). A sample of 82 mother-infant pairs (41 per randomised group) would allow the detection of a 3.13 points difference in perceived stress measured by PSS between groups at 80% power with a significance level of 0.05. To allow for potential drop-outs or failed measurements, a total sample of 120 infants was planned.

4.5.2. Data handling

The data collection was monitored by the researcher's supervisors during the study. Data that were found to be ambiguous were highlighted and double checked by the

researcher and supervisor. Participating mothers were contacted for clarification of odd values.

4.5.3. Statistical analysis of the main findings

Statistical analysis was conducted using SPSS (version 26.0). Intention-to-treat analysis was performed to compare primary outcomes (perceived stress and anxiety, infant weight and length gain from baseline to the endpoint) between intervention and control groups at the 8-week postpartum visit using two sample t-test. Paired t-tests were used to compare baseline to post-intervention changes of each primary outcome. The association between the effects of the intervention and the frequency of use was examined by two-tailed Pearson correlations. Two-sample t-tests were used to compare milk volume, energy and macronutrients level between intervention and control group. Regression analysis was used to adjust for confounding factors.

Furthermore, associations between infant temperament/behaviour and the composition of microbiota in mother's breastmilk, mother and infants' gut were examined using univariate ANOVA. Multiple regression analysis and MANOVA were used to assess differences among significant predictor variables identified by ANOVA. The significance level was set at $p < 0.05$ for all outcomes. However, p values between 0.05 and 0.1 were regarded as indicating a trend in other assessed outcomes. More details are provided in the Chapter 5 and Chapter 6 for baseline and outcome results.

4.5.4. Ethical considerations

Full ethical approval was obtained from both the research ethics committees of Beijing Children Hospital (2018-167) and University College London (12681/002) (Appendix 2). All participants provided written informed consent. During the recruitment the researcher checked the participants' understanding of the information sheet and ensured that they understood that their individual details and responses will remain confidential. Mothers were informed that if they did not participate, they would still receive the standard care from healthcare professionals and they could withdraw from the study at any time without any effect on their children.

The randomisation was undertaken by stealth as the mothers did not know about the randomisation to relaxation intervention/standard treatment until the end of the study. This was necessary because it is likely that mothers assigned to the standard treatment group might otherwise have used a relaxation intervention. We considered that this was acceptable, and it was approved by the research ethics committees, since all mothers received the same breastfeeding support so the control group were not deprived of any treatment. The intervention is a simple, non-invasive, very low-risk therapy which is an 'extra', so all mothers receive current standard-of-care treatment.

The test-weighing method used in this study has the potential to make the mother worried about her infant's weight or the amount of milk she is producing. To avoid this, the researcher explained that the results of test-weighing were intended only to give a

'snap shot' of the situation for an individual infant and that although they are useful for comparing groups of infants in a research study, they cannot be used alone to draw conclusions about the overall milk intake of an individual infant.

All mothers received a gift of a hand pump and an electronic gift card valued 200 CNY (about 23 GBP), as a token of appreciation for their participation. The pump was used to express milk during the study. All mothers also received individual consulting opportunities during the study period through personal chatting in Wechat. This information was provided in Participant Information Sheet (Appendix 4).

4.6. Summary

This chapter has presented the study framework and methodologies of the proposed RCT. Detailed statistical analyses are shown in corresponding chapters before presenting the results. In the next chapter, I present the baseline characteristics of the RCT with comparisons to the general population in Beijing and in China.

Chapter 5

Baseline Results of the Randomised Controlled Trial

This chapter provides descriptive results of the study population at baseline. The planned sample size was 120, but due to the impact of Covid-19recruitment could not be conducted during the lockdown period in Beijing (from 26th January to 31st July 2020) and it had to finish by the end of October 2020 due to the time constraints of the PhD. Therefore, a total of 96 mothers were recruited for the RCT. The population is categorised into two groups: 'late preterm (LP)' and 'early term (ET)'. The LP group comprised mothers of infants whose gestational age was 34 0/7 to 36 6/7; whereas the ET group comprised those whose gestational age was 37 0/7 to 37 6/7. Results presented in this chapter are: i) the characteristics of the mother-infant dyads and comparisons with national data [213] and local term and late preterm data from Beijing [214, 215]; ii) birth experiences and infant feeding practices of the mothers; iii) differences between the LP and ET groups.

Other primary outcomes regarding the effects of the intervention on maternal psychological status and infant growth are presented in the next chapter.

5.1. Study Population and Data Collection

Recruitment for this RCT started in February 2019 and ended in August 2020. The data collection was completed by the end of October 2020. During the recruitment stage, a total of 216 potentially eligible mothers were screened from the clinical records of four study centres (as mentioned in Chapter 4, Figure 4.1). A total of 178 eligible mothers were approached after birth, and 96 of them (54%) provided informed consent and were enrolled in the study. Of the remaining 82 mothers, 34 (41%) lost contact after discharge from the hospital, 28 (34%) infants had severe neonatal illness, 11(13%) mothers did not want to EBF at all, and 9 (11%) mothers refused to participate due to other health-related issues. Details of the study process are presented in **Figure 5.1**.

As described in Chapter 4(4.5.3), demographic information, maternal early hospital experiences and information on early feeding practices were collected using a socio-demographic questionnaire. IIFAS and BEBQ were used to assess maternal attitude towards breastfeeding and infant eating behaviours at baseline. Data are presented in total and separately for women in LP and ET groups. Descriptive data are shown as number (percentage) with the group differences tested by Chi-square. Continuous variables were checked for normality by using Q-Q Plots and histograms. For normally distributed data, mean ± standard deviation (SD) was presented along with the group differences tested by using the Student's T-test or ANOVA. Alternatively, median ± interquartile range (IQR) was presented along with the non-parametric test (Mann-Whitney or Kruskal-Wallis test) result.

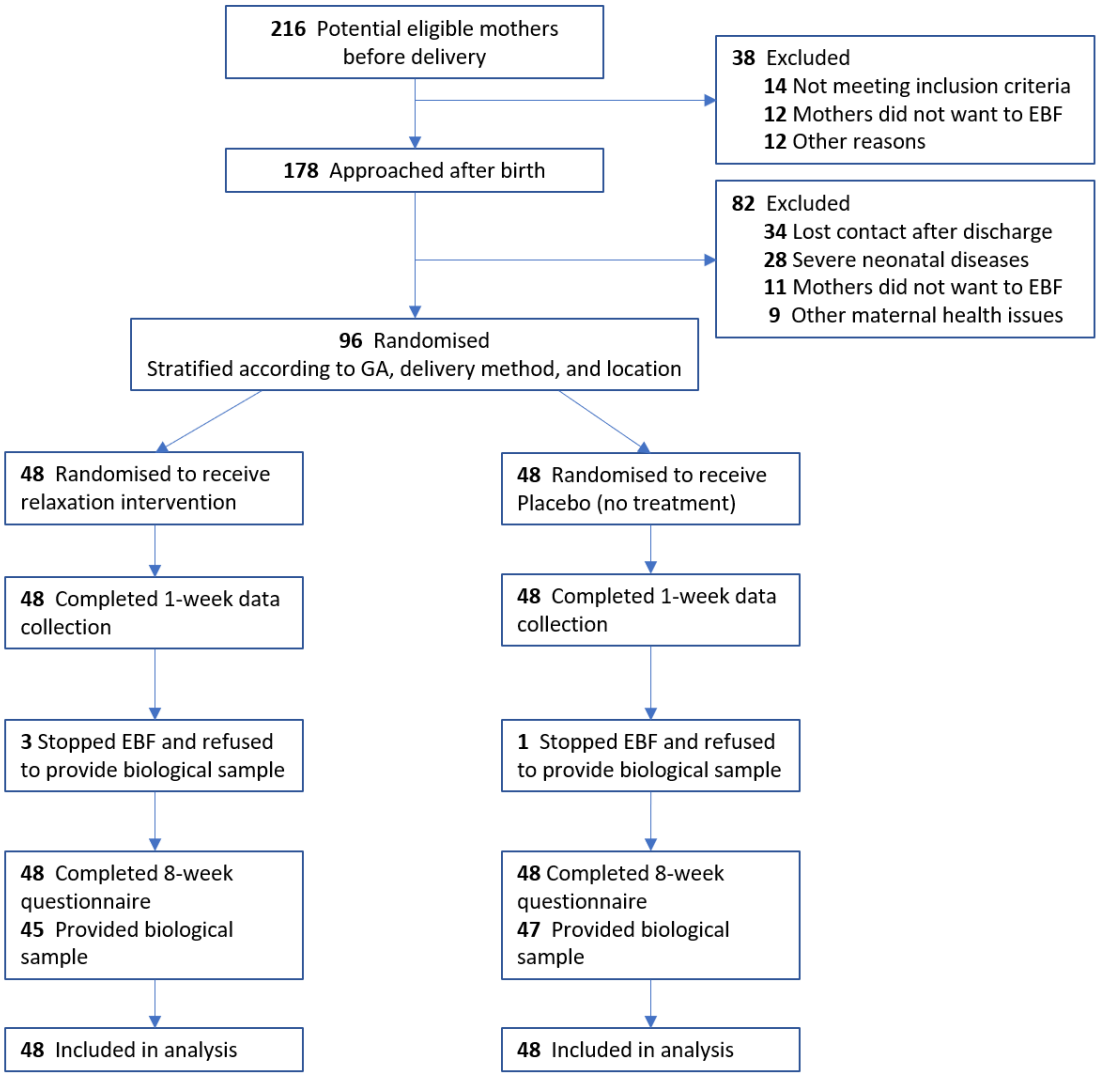


Figure 5.1. Flow chart of the randomised controlled trial
Notes: GA= gestational age; EBF=exclusive breastfeeding

5.2. Demographic Characteristics of the Study Population

Table 5.1 shows the socio-demographic characteristics of the study population and the comparisons with national and local data. The national data used for comparison with the current study data were extracted from a large cohort study in China which include 10,408 mother-infant dyads who participated in a survey on factors influencing breastfeeding in 2017-2018 [30, 213]. For late preterm and term infants, I compared my results with the findings from a local study that included 1576 late preterm infants who were hospitalized in the neonatal wards of 25 hospitals in Beijing in 2015-2017 [215]. Moreover, as shown in Table 5.1, results from this study were also compared with data from the COVID-19 New Mum study in Beijing, which included 2103 term and 96 preterm infants in 2020 [214]. The baseline results also compared with international data which are presented in the discussion.

Overall, 11.5% of the participants lived in central Beijing (within the Five Ring Road of Beijing). No significant differences were found between LP and ET groups for demographic characteristics within the whole study population.

Table 5.1. Baseline characteristics of the included mothers and infants at 1- week postpartum.

Descriptive characteristics	Total (all)		LP	ET	(LP vs. ET)	National data [c]	Beijing Data [d]	Beijing preterm [e]
	(n=96)		(n=57)	(n=39)	p-value	n=5112	n=2233	n=153
	N	%	N (%)	N (%)		%	%	%
Baby's gender					0.680			
Male	52	54.2	32(56.1)	20(51.3)		50.2	50.6	51.6
Female	44	45.4	25(43.9)	19(48.7)		49.8	49.4	48.4
Location of recruitment					**<0.001**			
Northeast Beijing (Centre A)	39	40.6	18(31.6)	21(53.8)				
Central Beijing (Centre B)	11	11.5	2(3.5)	9(23.1)				
Northwest Beijing (Centre C)	21	21.9	16(28.1)	5(12.8)				
South Beijing (Centre D)	25	26	21(36.8)	4(10.3)				
Maternal age group (years)					0.290[a]			
20-25	8	8.2	4(7.0)	4(10.3)		25.2	6.2	
26-30	53	54.6	28(49.1)	25(64.1)		63.3	36.6	
31-35	29	29.9	20(35.1)	9(23.1)		-	40.9	
>35	6	6.2	5(8.8)	1(2.6)		11.4	16.3	
Educational levels					0.809			
College and under	29	30.3	15(26.4)	12(30.8)		78.6		
Bachelor degree	54	56.3	34(59.6)	22(56.4)		21.4	42.1	
Postgraduate	13	13.5	8(14.0)	5(12.8)		-	15.8	
Household income (CNY/year)					0.274[a]			
<200,000	40	41.7	25(43.9)	13(33.3)			33.3	
200,000-300,000	31	32.3	21(36.8)	11(28.2)			24.4	
300,000-450,000	12	12.5	7(12.3)	5(12.8)			13.1	
>450,000	13	13.5	4(7.0)	10(25.6)			7.7	
Birth hospital					0.063[a]			
Public hospital	84	87.5	53(93.0)	31(79.5)		90.1		
Private hospital	12	12.5	4(7.0)	8(20.5)		9.9		

Main maternity care person					0.727[a]
Husband	60	62.5	35(61.4)	25(64.1)	
Parents	24	25	16(28.1)	8(20.5)	
In-laws	9	9.4	5(8.8)	4(10.3)	
Confinement lady	3	3.1	1(1.8)	2(5.1)	

Notes:[a] Monte Carlo was used due to small expected counts in CrossTabs. [b] Tested by using independent t-test.

[c]. [213]. [d] [214]. [e] [215]. LP=late preterm; ET=early term; CNY=Chinese Yuan.

Male infants accounted for 54.2% in this study, with no significant difference between LP and ET groups. The majority of infants were born at 36-37 weeks (77.1%). The mean gestational ages were 36.1±0.9 and 35.5±0.7 for all infants and for LPs respectively. All mothers included in this study were married. The mean age of the mothers was 30±3.4 years with the majority in the 26-35 years group. No significant differences were found in maternal age between the LP and ET groups, (30±4 years and 30±3 years respectively). The distribution of LP and ET mothers from the four study centres varied; study centre D had significantly more LP mothers and ET mothers, since the hospital in centre D is the largest preterm infant hospital in China.

Most mothers had a Bachelor's degree or above (69.8%). Compared to the ET mothers, significantly more mothers of LPs lived in Northwest and South Beijing, where the annual household income was mostly less than 300,000 CNY. Significantly more mothers of ET were in the highest annual income group compared to LP mothers (25.6% vs.7%, p=0.028). Moreover, the mean infant length at discharge was 46.9±1.9 and 48.8±1.3 for LP and ET respectively, whilst mean weight was 2622±309 grams and 2830±277grams.

Compared to national and local data, a similar gender ratio was found with 50.2%-50.6% male term infants. For LP infants, data were compare to a local late preterm study where the mean gestational age was similar to my study (35.4±0.8) [215]. Male participants in the local LP study accounted for 51.6% and 53.8% of breastfed and formula fed infants respectively. Infant weight and length at the 1-week home visit were compared with the local and national data which are assessed at discharge (normally 4-10 days for healthy

preterm infants). Compared to my results, the local preterm data reported lower infant weights at discharge, with weights of 2370 (SD=418) grams and 2478 (SD=414) grams for breastfed and formula fed infants respectively. My length data were consistent with the local late preterm study, with lengths of 46.8 (SD=3.4) and 47.1 (SD=2.6) cm for breastfed and formula fed infants respectively (**Figure 5.2**).

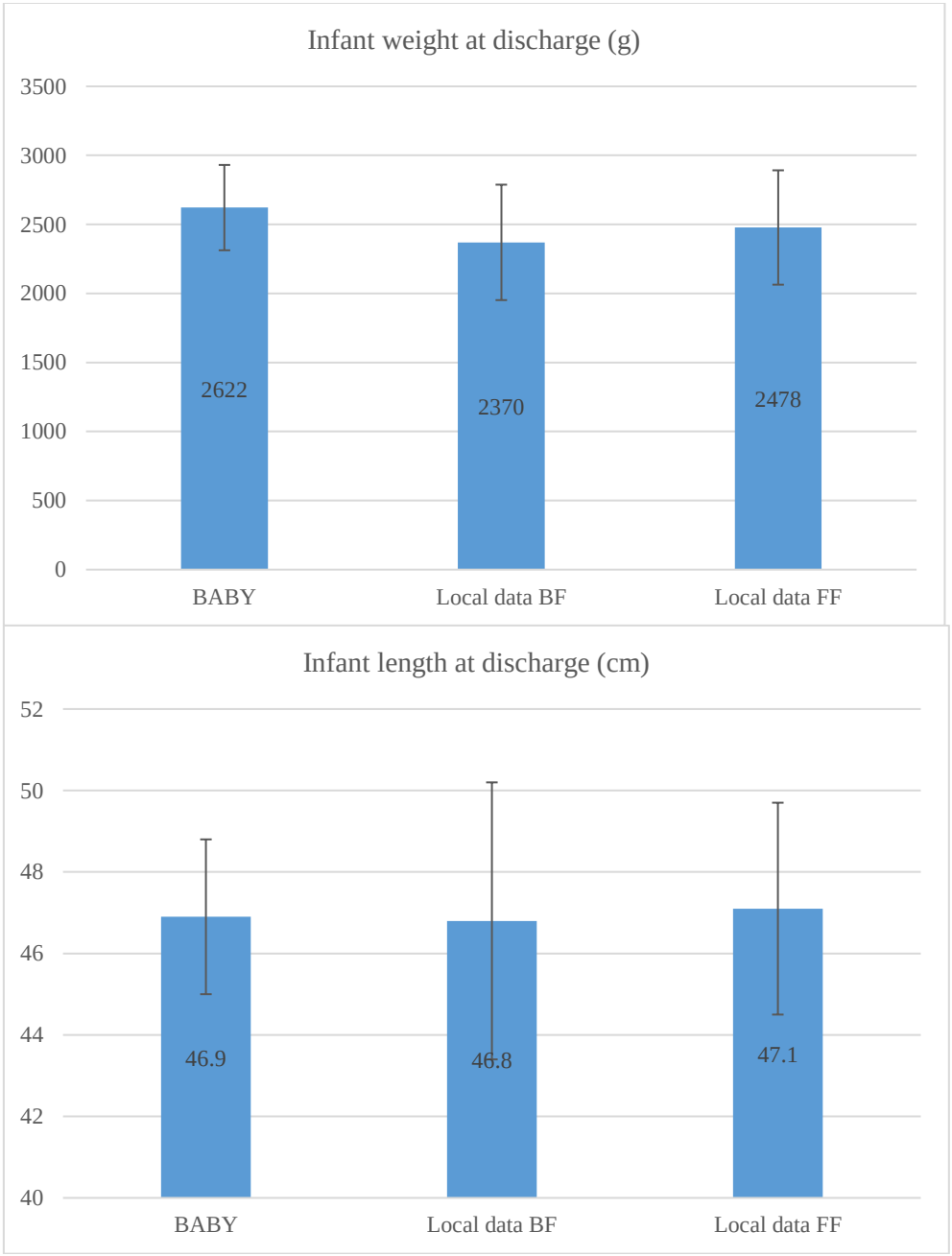


Figure 5.2. Comparisons of infant weight and length at discharge with local data.
Notes: BF=breastfeeding, FF=formula feeding.

5.3. Birth Experiences

As shown in Table 5.2, no significant difference was found in early birth experiences between LP and ET groups. More mothers in the study gave birth at public hospitals rather than private hospitals. Over half of the mothers delivered vaginally (25% not induced, 38.5% induced). While mothers aged between 20-25 years old showed the highest rate of caesarean (62.5%); mothers aged between 26-30 presented the lowest rate (32.1%). However, the association between maternal age and delivery method was not significant (p=0.08). The caesarean rate was also not significantly different between male and female or LP/ET. Almost half of mothers did not use any pain relief during labour mainly due to preference for a natural birth. Of the 21.1% mothers requiring an instrumental delivery, 47.1% were delivered using the vacuum aspirator and 52.9% using forceps. Mothers of LPs were more likely to require an instrumental delivery and had a longer hospital stay than ET mothers, but the difference between groups was not significant.

Skin-to-skin contact after delivery is defined as placing the naked baby on the mother's bare abdomen or chest immediately or less than 10 minutes after birth or soon afterwards [216]. After birth, more ETs than LPs had skin-to-skin contact with their mothers immediately or within the first 30 minutes. A small number of LP mothers did not have contact with their infants within the first hour after birth, whilst all ET mothers had skin contact with their infants within the first hour. Besides, over half of mothers reported that the skin-to-skin contact was less than 20 minutes. As for the main

maternity care person, the majority of mothers chose their husband, followed by their parents and their parents-in-law.

Table 5.2. Early postnatal experience of the mothers

	LP		ET		Total		p	Reference [b]
	n	%	n	%	n	%		n (%)
Mode of delivery							0.487[a]	
Vaginal, not induced	25	43.9	12	30.8	37	38.5		838(38.76) [b]
Vaginal, induced	10	17.5	14	35.9	24	25		32 (1.48) [b]
Planned caesarean	5	8.8	4	10.3	9	9.4		114(5.27) [b]
Unplanned caesarean	17	29.8	9	23.1	26	27.1		1178(54.49) [b]
How soon did skin-to-skin contact occur after delivery?							0.537	
Directly after birth	15	26.3	16	41	31	32.3		
About 15-30 mins after birth	22	38.6	16	41	38	39.6		
More than 30 mins after birth	20	35.1	7	18	27	28.1		
How long was the skin-to-skin contact after birth?							0.568	
Less than 20 mins	33	57.9	26	66.7	59	61.5		18(40) [c]
20 mins to 1 hour	13	22.8	13	33.3	26	27.1		26(57.8) [c]
Not within 1 hour after birth	11	19.3	0	0	11	11.4		1(2.2) [c]
Did midwifery used during the labour							0.789	
No	45	78.9	34	87.2	79	82.3		
Yes	12	21.1	5	12.8	17	17.7		
Medication during labour							0.249	
Spinal/Epidural	22	38.6	12	30.8	34	35.4		
Other pain medication	9	18.8	11	28.2	20	20.9		
None medication	26	45.6	16	41	42	43.8		
First feeding after birth								
Less than 30 mins	9	15.8	9	23.1	18	18.8	**0.048**	
30 mins-2 hours	9	15.8	13	33.3	22	22.9		
2-24 hours	13	22.8	9	23.1	22	22.9		
More than 48 hours	26	45.6	8	20.5	34	35.4		
Hospital stays after birth							0.578[a]	
Less than 48 hours	7	12.3	3	7.7	10	10.4		

48-72 hours	12	21.1	12	30.8	24	25
More than 72 hours	38	66.7	24	61.5	62	64.6

Notes: LP=late preterm; ET=early term. [a] Monte Carlo was used due to small expected counts in CrossTabs. mins=minutes [b] [217]. [c] [218]

5.4. Early Feeding

5.4.1. Early feeding practices

The early feeding practice for the 96 included mothers was investigated using the self-reported questionnaire. Mothers were asking when the first feeding occurred after birth, if their infants had been fed with other fluid before they enrolled in the study, and their current feeding method at baseline. Overall, 64.6% mothers reported that the first feeding occurred within 24 hours after birth. More mothers in the LP group started feeding later than 48 hours after birth, compared to ET mothers (45.6% vs. 20.5%). Almost a half of the infants (43.6%) had been fed with formula or other liquid before they enrolled in the study. For this study, mothers were defined as EBF if they chose "exclusively breastfeeding" as their current feeding method and if they also chose "no" as the answer to the question "has your infant ever received any other fluid apart from breast milk?". Milk fortifier for preterm infants and expressed breast milk were included as "EBF" while water and other fluid were not included; details were explained by the nurse who collected the data. Using this definition, the EBF rate[1] at baseline (5-10 days after birth) was 56.4% for all mothers, and 52.6% versus 61.5% for LP and ET groups respectively (X^2=2.564, p=0.278).

[1] If mothers had fed the infant with other fluids initially, as long as they were EBF at the baseline visit, they were eligible for inclusion in the trial. However hereere 'EBF' refers to mothers who were exclusively breastfeeding from delivery to the baseline visit.

5.4.2. Maternal breastfeeding attitudes

Maternal attitudes toward breastfeeding were assessed by IIFAS. Mothers rated their attitudes toward each question from 1 (strongly disagree) to 5 (strongly agree). Questions favouring formula feeding were reverse-scored, and the total score was calculated by the sum of each score. Total attitude scores could range from 17 (indicating negative attitudes toward breastfeeding feeding) to 85 (reflecting positive attitudes towards breastfeeding), while a score of 51 indicated a neutral attitude.

Table 5.3 shows the mean score of each individual item and the total score for mothers of LP and ET groups. The average population mean score is 62.1, which indicates a neutral to positive attitude toward breastfeeding. No significant differences were found in total IIFAS score or any individual items between LP and ET groups. Results of the Pearson correlation showed that the IIFAS score was significantly positively associated with the total years of maternal full-time education ($r=0.28$, $p=0.006$), academic degree ($r=0.21$, $p=0.043$), and family income ($r=0.21$, $p=0.042$). Furthermore, a higher IIFAS score was also significantly related to earlier skin-to-skin contact ($r=0.20$, $p=0.048$) and earlier time for first feeding after birth ($r=0.22$, $p=0.033$). The IIFAS score was higher in mothers who had skin-to-skin contact immediately after birth (mean IIFAS score=63.9) and who started feeding in the first 30 minutes (mean IIFAS score=63.6) compared to mothers whose skin-to-skin contact occurred after 30 minutes (mean IIFAS score=60.2) and mothers who started feeding later than 48 hours (mean IIFAS score=60.5), however, the difference was not significant. One-way ANOVA was performed to ascertain the

differences of IIFAS score between educational level and family income category of the mothers. Results showed that there was a significant difference in IIFAS score according to the family income category (F=3.531, p=0.018). The post-hoc (Bonferroni) results suggested that mothers whose annual family income was around 300,000-450,000 CNY had significantly higher IIFAS mean score than those whose annual income was less than 200,000 CNY. Moreover, EBF mothers had higher IIFAS score than those whose infants had been fed with formula at baseline (63.3 vs. 59.4, p=0.07).

When assessing the LP and ET mothers separately, the total IIFAS score was significantly related to maternal full-time education in LP mothers (r=0.27, p=0.039) and ET mothers (r=0.33, p=0.042), and was significantly correlated with the starting time of first feeding in LP mothers (r=-0.27, p=0.045), but not ET mothers. There were no differences in the associations with the other factors between LP and ET groups.

Table 5.3. Maternal attitudes towards breastfeeding at 1-week postpartum.

Items	All (n=96)		LP (n = 57)		ET (n=39)		t-test		
	Mean	SD	Mean	SD	Mean	SD	P-value	95% CI	
1. The nutritional benefits of breast milk last only until the baby is weaned from breast milk. ǂ	3.28	1.32	3.16	1.32	3.46	1.31	0.27	-0.85	0.24
2. Formula-feeding is more convenient than breastfeeding. ǂ	3.57	1.07	3.56	1.05	3.59	1.12	0.90	-0.47	0.41
3. Breastfeeding increases mother-infant bonding.	4.41	1.07	4.46	1.04	4.33	1.13	0.58	-0.32	0.57
4. Breast milk is lacking in iron. ǂ	3.77	0.85	3.72	0.84	3.85	0.87	0.48	-0.48	0.23
5. Formula-fed babies are more likely to be overfed than breast-fed babies.	3.50	1.06	3.53	1.02	3.46	1.12	0.77	-0.37	0.50
6. Formula-feeding is the better choice if a mother plans to work outside home. ǂ	3.22	1.03	3.19	1.08	3.26	0.97	0.77	-0.49	0.36
7. Mothers who formula-feed miss one of the great joys of motherhood.	3.90	1.18	3.74	1.28	4.13	1.00	0.11	-0.88	0.09
8. Women should not breast-feed in public places such as restaurants. ǂ	3.27	1.29	3.39	1.26	3.10	1.31	0.29	-0.25	0.81
9. Babies fed breast milk are healthier than babies who are fed formula.	4.06	1.00	4.02	1.06	4.13	0.92	0.60	-0.53	0.31
10.Breast-fed babies are more likely to be overfed than formula-fed babies. ǂ	3.64	0.94	3.61	0.88	3.67	1.03	0.79	-0.44	0.34
11.Fathers feel left out if a mother breast-feeds. ǂ	3.44	0.99	3.46	1.00	3.41	0.99	0.83	-0.28	0.46
12.Breast milk is the ideal food for babies.	4.50	0.86	4.49	0.87	4.51	0.85	0.90	-0.37	0.33
13.Breast milk is more easily digested than formula.	4.33	0.99	4.32	1.05	4.36	0.90	0.84	-0.38	0.37
14.Formula milk is as healthy for an infant as breast milk. ǂ	2.99	1.07	3.04	1.10	2.92	1.04	0.62	-0.45	0.56
15.Breastfeeding is more convenient than formula feeding.	3.88	0.95	3.75	0.97	4.05	0.92	0.14	-0.68	0.09
16.Breast milk is less expensive than formula.	4.08	1.03	4.12	1.02	4.03	1.06	0.65	-0.33	0.53
17.A mother who occasionally drinks alcohol should not breast-feed her baby. ǂ	2.27	1.22	2.11	1.10	2.51	1.35	0.11	-0.91	0.09
Total scores of IIFAS	62.10	6.74	61.65	6.52	62.77	7.07	0.43	-3.90	1.66

Notes: LP=late preterm, ET=early term. * p-value < 0.05; ǂ Unfavourable to breastfeeding (reversed score applied). CI=confidence interval.

5.5. Discussion

A total of 178 mothers were approached during recruitment, while 82 of them were not eligible to be included in the study. A total of 34 mothers lost contact after discharge from the hospital; the nurses reported that most of them went back to their hometown (outside Beijing) and were therefore unable to participate, though they expressed an interest before delivery. Moreover, 37 (45.1%) of the excluded participants were not able to participate due to severe neonatal illness or maternal health issues that led to a long period of mother-infant separation. The age for those mothers ranged from 29 to 37 years (mean=34.7), and most had gestational diabetes and/or gestational hypertension, consistent with a higher risk of having health issues after birth for both mothers and infants in older primiparous women [219, 220].

Beijing is the biggest city in China. There are 16 districts (counties) under the jurisdiction of the city, with a total area of 16410.54 square kilometres. As reported by the National Bureau of Statistics, the resident population of Beijing was over 21 million in 2020, while nine million residents were immigrants from outside Beijing [221]. To make the study population more representative, the recruitment was conducted in four different areas of Beijing, including central Beijing, northeast, northwest, and south Beijing. As shown in Figure 5.2, study centre A was located in Shunyi District, where the gap between rich and poor is large; since there are both rural villages and luxurious villas in this district. The large population and the diverse social-class of residents makes this district the most popular area for population studies in China; it was also the area where recruitment of

the Chinese part of the Intergrowth-21st Project was conducted [222]. Study centre B was located in central Beijing, where the collaboration hospital, Haidian Maternal and Child Health Hospital, is ranked second for annual birth numbers in Beijing [221]; residents in central Beijing were mostly recruited from this hospital. Centre C was located in the largest community, Huilongguan community in Changping District, where 85.4% of the residents were immigrants from outside Beijing [223]. Participants from centre D were living in Daxing Disctrict of Beijing, and represented the population living in south Beijing; the collaboration hospital for centre D was one of the largest hospitals for preterm birth in China. Given these characteristics, the study population included women with a wide range of backgrounds that would overall be representative of Beijing women.

The baseline demographic characteristics were consistent with the local data (n=2233) for maternal age, education level, infant gender ratio, and gestational age. However, mothers in the present study were more educated compared to the age-matched national population, as women with a Bachelor degree accounted for 69.8% in the present study and 21.4% in national data (n=5112).While this may be due to the fact that the general education level in Beijing is higher than national data [224], it also seems likely that more highly educated mothers might have a greater interest in breastfeeding research and be more willing to follow the quite complicated and demanding study protocol.

As shown in a retrospective cohort study in Beijing (n=30977) [217], the rate of caesarean delivery for all infants was highest at 60.7% in 2002 and declined to 34.5% in 2014. Similarly, in the COVID-19 study in Beijing, the rate of caesarean section (CS) was 34.4% in 2020 (n=2233). The gestational age of infants in these two studies were both >28 weeks while the majority of infants were term infants. Results of these two studies were consistent with the caesarean rate of term infants in my study. However, in another late preterm study which included n=1608 LP mother-infant pairs from 25 hospitals in Beijing [215], the caesarean rate was 64.2% and 67.3% for formula and breast-fed infants respectively, which was higher than in my study. The national data suggests that the rate of CS differs according to the region. Results from a large birth cohort study in China showed an increased caesarean rate from 28.8% in 2008,34.9% in 2014 and 36.7% in 2018; moreover, the caesarean rate varied from more than 60% in some supercities to less than 10% in some rural areas [225]. For LP birth, a systematic review showed the CS rate was 40.4% in rural or undeveloped regions of China [226], whilst another study showed a higher CS rate of 64.9% in Zhejiang, which is the most developed and richest province in China [227]. Consistently, a systematic review showed that the caesarean rate was higher in urban than in rural areas. These figures suggest the CS rate tends to be higher in more developed regions than in rural areas of China. In fact, this applies not only to China, as WHO data on the CS rate in South Asia showed that the highest CS rates are in more developed cities and the overall caesarean rate was 18.1% in 2015 [228]. As suggested by Zhang et al. [229], the factors influencing the CS rate included previous caesarean delivery (accounting for 38.2% of all caesarean

deliveries), maternal request (9.8%), labour dystocia (8.3%), fetal distress (7.7%) and malpresentation (7.6%), whilst Akhter et al.[230] reported that urban area mothers appeared to have higher educational status compared to those in rural areas, which offers them independence as well as more control over their birth plan; however, better education may not provide them with better knowledge of the risks of caesarean delivery.

Moreover, data from a retrospective cohort which analysed 233,844 deliveries among 19 hospitals across the United States between 2002 and 2008 showed that the CS rate for LP infants were 38.3% while for term infants it was 27.2% [231]. More recently, a US national study showed an average of 32% CS rate for all births in 2015 among counties[232]. While the average CS rate was similar to that in several studies in China, it should be mentioned that this is considerably higher than the 10%-15% recommended by the WHO [228]. As suggested by Maeda et al. [233] , higher caesarean rates can be associated with limited or unconsolidated medical resources. Accordingly, policymakers should be aware of regional differences and the possible effects of perinatal care resources on caesarean rates.

Maternal early birth experience is associated with postpartum recovery and infant development [234, 235]. Research shows that if the mother sees, feeds and has skin-to-skin contact with their infant in the first hour after birth this could trigger the process of internal development of the newborn, which plays a key role in early coordination of the five senses of the infant (sight, hearing, touch, taste, and smell) [236]. Experimental

studies also suggested that skin contact may promote breast milk secretion by increasing the oxytocin levels in mothers, and may also be related to maternal behaviour and bonding after birth [235, 236]. A systematic review reported that mothers who have skin-to-skin contact within the first hour after birth are more likely to have longer breastfeeding, and exclusively breastfeed up to six months after birth; while infants who receive skin contact in the first hour may have better stability of the cardio-respiratory system, and higher blood glucose levels [237]. WHO recommends the practice of skin-to-skin contact for at least one hour after birth, and health care providers should encourage women to recognise when their babies are ready to breastfeed and offer help if needed [238]. However, currently there is lack of data on the skin-to skin contact rate in late preterm infants [237]. For term infants, the rate varies among countries. A systematic review showed the skin-to skin contact rate was estimated to be between 72% and 95% based on current Australian studies [237]. In South Korea, a retrospective study reported that 76% mothers experienced skin-to skin contact for the first 30 minutes after birth [239]. In Europe, a national study in Denmark assessed 269,597 births showing that 96% of women had skin-to skin contact within the first 2-hours after birth, whilst a study conducted in eight hospitals in France [240]and a study conducted in London, UK [241] reported a similar rate of skin-to skin contact within the first 2-hours after birth at 64%.

In my study, all mothers of ETs had skin-to skin contact in the first hour after birth, and 82% started the contact within 30 minutes. However, 11 of the 57 LP mothers (19.3%)

did not have skin-to-skin contact within the first hour, since their infants were transferred to the NICU for medical reasons; in this context, some studies investigated the effects of delivery room skin-to-skin contact (DR-SSC) on healthy preterm infants' development. An RCT in a level III Germany NICU assessed the effects of 60 minutes DR-SSC on healthy preterm mother-infant interaction, maternal depression, stress and bonding at 6 months corrected age [242]; results showed a higher quantity of mother-child interaction, and lower risk of early postpartum depression (15% vs 45%, p=0.003) and impaired bonding (p=0.031) compared to the 5 minutes visual contact group. Another RCT in 88 healthy singleton preterm infants reported that a 60 minutes DR-SSC results in significantly altered stress response gene expression which may contribute to improved outcome of prematurity [243]. These findings suggested the beneficial effects for promoting 60 minutes DR-SSC in clinical settings for healthy preterm mothers and infants. It should be mentioned that both studies discussed above excluded infants with severe malformations and syndromic disorder or infants who need resuscitation or had cardiopulmonary failure after birth.

Moreover, in my study, all 11 mothers that did not have skin-to-skin contact within the first hour also reported a later first breastfeeding (after 48 hours); although some of them provided expressed breast milk to their infants, which may contribute to the result that the feeding started significantly later in mothers of LP than ET. Early skin-to-skin contact was associated with successful early initiation of breastfeeding [244], hence mothers of preterm infants may face more challenge regarding establishing

breastfeeding. Given this, in clinical settings, mothers of preterm infants need special attention and support for the establishment of breastfeeding.

Additionally, when assessing maternal attitudes and perceptions toward breastfeeding in my study population, the general attitude was neutral to positive, which was consistent with a previous study performed in Beijing mothers (n=45) [218]. No significant differences were found in total IIFAS score between LP and ET mothers. Pearson correlation results show that the total score of IIFAS was significantly related to the starting time of first feeding and skin-to-skin contact. The IIFAS score was higher in mothers who had skin-to-skin contact immediately after birth compared to mothers whose skin-to-skin contact occurred after 30 minutes. Although this difference was not significant, it was consistent with previous research suggesting the importance of early skin-to-skin contact in successful initiation of breastfeeding, breastfeeding confidence, and longer breastfeeding [235, 237, 244]. Moreover, a structured survey with two measurement points in Finland showed although the IIFAS score in the study population was generally positive immediately after birth, it decreased during their infants' hospital stays [245]; besides, the study found a breastfeeding-favourable attitude and early physical contact can predict earlier initiation of breastfeeding and the frequency of breastfeeding. These findings highlight the need to support mothers before discharge, to help them build and maintain confidence in breastfeeding.

5.6. Conclusion

This chapter outlined the socio-demographic characteristics and the early experiences after birth in the study population. Maternal characteristics and delivery experience after birth were compared between late preterm and early term groups, and also compared to the general population in Beijing and national wide. Overall, although the CS rate is higher in my study compared to international data, the early experiences of mothers in my study were reasonably good in terms of early initiation of breastfeeding and skin to skin contact. The study population was generally representative except the education of mothers was higher than the national population. This is considered in later chapters when generalising the main outcomes of the study to the whole population of China, given there is a positive association between maternal education level and attitudes toward breastfeeding.

In the next chapter, the primary outcomes of the randomised controlled trial are presented, with comparisons of baseline characteristics and primary outcomes between relaxation (n=48) and control groups (n=48).

Summary points:

- The study population had similar socio-demographic characteristics to the general population of mothers in Beijing, but were more educated than the Chinese mothers in general.

- The late preterm and early term mothers had similar characteristics and demographic backgrounds, except that significantly more late preterm mothers were recruited from south Beijing.

- For both groups of mothers, the main primary maternity care person was their husband.

- Both late preterm and early term mothers had similar perceptions towards breastfeeding, indicating a neutral to positive attitude.

- The attitude towards breastfeeding was significantly positively correlated with maternal education, family income, earlier skin-to-skin contact and earlier time of first feeding after birth.

Chapter 6

Primary and Secondary Results of Randomised Controlled Trial

The primary outcomes and most secondary outcomes of the randomised controlled trial (RCT) are presented in this chapter, including the following components:

I) Baseline results and comparisons between intervention (IG) and control groups (CG): descriptive characteristics of mothers and infants, breastfeeding attitudes and maternal postpartum experiences.

II) Primary outcomes of the RCT:
    i) changes in maternal stress and anxiety from 1-week to 8-weeks;
    ii) infant weight and length gain from 1-week to 8-weeks.

III) Main secondary outcomes of the RCT:
    i) macronutrient content of breast milk: fat, protein, carbohydrate at 8-weeks;
    ii) breast milk volume and milk energy at 8 weeks;
    iii) maternal breastfeeding attitudes and infant appetite at 8-weeks;
    iv) infant behaviour measured by 3-day infant behaviour diary at 8-weeks.

Moreover, I have conducted additional analysis to explore interactions between the intervention and infant gender/gestational age, the association between maternal

stress and infant behaviour, as well as the dose-response effects of the intervention on primary outcomes and milk composition. These analyses, which were not planned in the original protocol, are outlined in section 6.5.6 "*Results of exploratory analyses*" of this chapter. Other secondary outcomes, including the composition and changes of microbiota in breastmilk, maternal and infant stool samples are provided in the next chapter.

6.1. Randomisation/control groups and follow-up visits

A total of 96 participants were randomly assigned to either intervention group (IG, n=48) or control group (CG, n=48) using sealed, opaque envelopes. To ensure that the number and characteristics of subjects assigned to each group was equally distributed, the randomization was stratified by infant gestational age (34-35 weeks, 36-37 weeks), delivery method (vaginal, caesarean), and by the study centre.

To minimise the risk that mothers in the control group might seek any type of relaxation therapy, all participants were blinded to the randomisation until the end of the study; they were told that the aim of the study was to investigate factors that may improve breastfeeding for new mothers. Detailed procedures of the randomisation process and follow-up data collections were outlined in **Figure 6.1**.

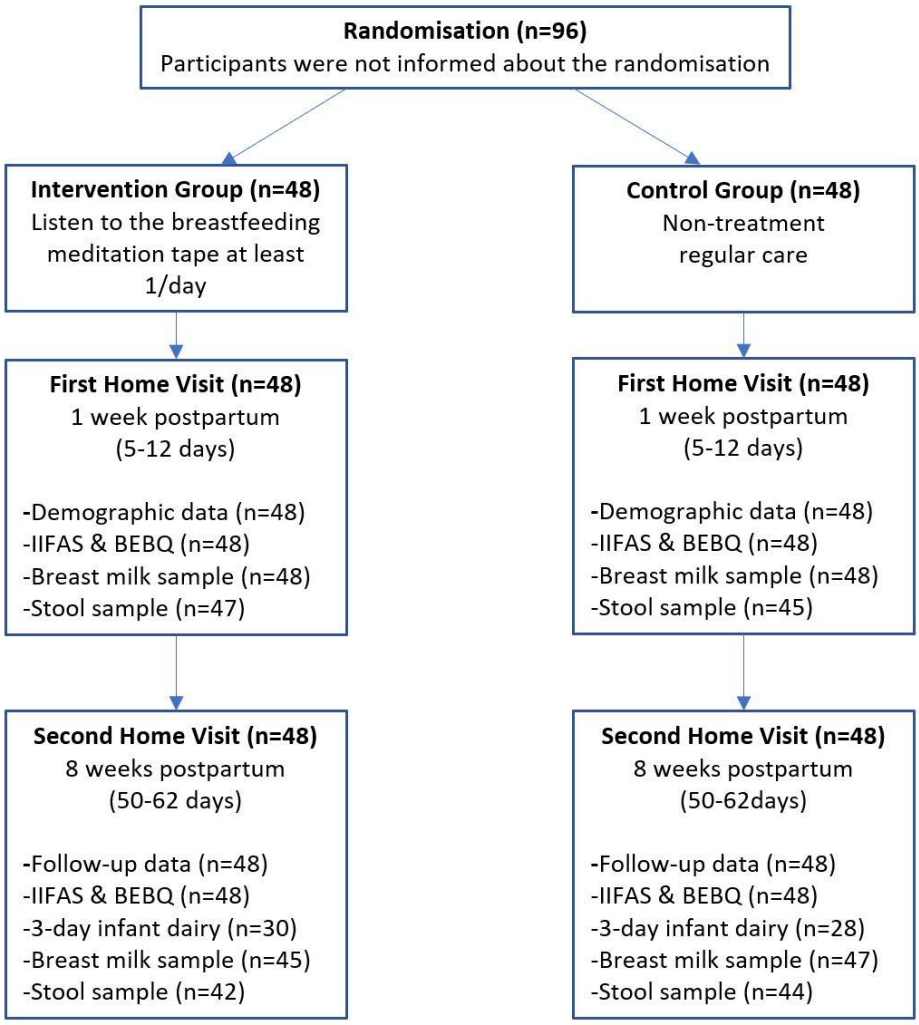


Figure 6.1. Flow chart of randomisation process and follow-up data collections.

Notes: IIFAS=IOWA Infant Feeding Attitudes Scale; BEBQ=Baby Eating Behaviour Questionnaire

6.2. Research methods

The research methods were described in Chapter 4. This section provides more detail on some specific issues during the data collection and sample analysis period. It also outlines the methods of the statistical analysis specifically for each outcome of the study.

6.2.1. Data collection

Home visits for data collection were conducted mostly around 10:00 a.m. in the morning. For mothers who were not available, the visit was re-arranged within 3 days. I went with two nurses from the nearest collaborating local clinic to the mother's home. According to the local clinic's regulation, only their clinical staff could touch the mother and baby during the home visit. Hence, my role during the home visit was to hold the materials (scale, cooler box, etc.) and supervise the nurses' work. **Table 6.1** provides details about the questionnaires and measurements used to assess the primary outcomes during 1-week and 8-week home visits. To ensure the study procedures could be standardised, all research assistants and nurses attended training, including the method for remote randomisation, the use and explanation of all study questionnaires, infant anthropometry, and the methods for collecting biological samples prior to the start of the study. Anthropometric assessment was conducted following the WHO instructions. All study centres used the same electronic scales (Betterren-FSG-25-YE, China) for the measurement of the baby's weight and length. The infant was weighed naked with a towel placed over the scale before resetting to zero. Infant length was measured to the nearest 0.1 cm on a recumbent board on the weight scale (Betterren-FSG-25-YE, China). A pre-test was conducted to ensure the accuracy of all scales; the scales at each centre were calibrated before and after each test.

Moreover, I participated in the biological sample collection and infant anthropometric measurements in person throughout all home visits at study centre B, C, and D, to

ensure the measurement standards were consistent. All biological samples were put in my car refrigerator after collection and I transported them immediately to the BCH laboratory (Lab). Due to transportation difficulties, I did not participate in the data collection in centre A, and the biological samples were temporarily stored at -80°C refrigerator in the Lab of centre A. However, I attended regular monthly meetings at centre A to emphasise the standardisation of measurement methods. After the meeting, I transported the biological samples to the BCH Lab.

6.2.2. Analysis of maternal psychological status

As stated in Chapter 4, maternal stress was evaluated using the Perceived Stress Scale (PSS) whilst the Beck Anxiety Inventory (BAI) was used to measure maternal anxiety. It should be acknowledged that compared to the PSS, which measures the perceived stress at different levels during daily life, the Beck Anxiety Inventory was originally used for measuring the severity of anxiety in psychiatric populations. Some symptoms described in the BAI might not be experienced by most healthy people (e.g. “feeling shaky or unsteady” “fear of dying”), which might lead to a generally lower score in all mothers, thus reducing the size of pre-post intervention differences. However, mothers in the present RCT were considered to have greater tension due to their LP and ET delivery, hence it was considered appropriate to use the BAI to identify the effects of intervention on their severity of anxiety. The use of both PSS and BAI also allowed comparison with the results from the MOM study which used the same tools.

6.2.3. Analysis of breast milk composition and energy content

Maternal foremilk samples were stored at -80°C in the Lab of BCH. Mothers provided three to four specimen jars of their foremilk with 4-5ml per jar. For the analyses of macronutrient composition in breastmilk, samples (4-5ml) were thawed at room temperature (27-29°C) and were then homogenised using the SX Sonicator (FS-T, SXSONIC, China). The samples were then measured by a near-infrared spectroscopy human milk analyser (MR-1011, HLIFE, China), after checking the calibration.

The milk analyser measures fat, total carbohydrate content (both lactose and oligosaccharide), protein (crude protein, with non-protein nitrogen), and total milk energy (kcal/100ml). The total milk energy of the breast milk sample is calculated by the milk analyser using the following equation: Energy kcal/100ml= (9.3 kcal/g * fat g/100ml) + (4.0 kcal/ g * protein g/100ml) + (4.0 kcal/g * carbohydrate g/100ml). The analyser was cleaned and calibrated by students at the Lab once a day. If odd values were obtained, for example, a value significantly higher or lower than the reference value, a duplicate analysis was conducted to check the results; calibration and cleaning was performed specifically for the checking.

6.2.4. Analysis of breast milk intake

Breast milk intake was assessed by 48-hour test-weighing as described in Chapter 4. The volume of milk intake was adjusted for 5% insensible water losses (IWL) based on published data [209]. The average milk intake in 24 hours was calculated by i) calculating

the volume of milk intake from each feed by subtracting the pre-feeding infant weight from the post feeding infant weight; ii) summing the volumes of each feed from the beginning of the first feed of the first day to the last feed on the last day; ii) dividing by the total feeding frequencies; iii) multiplying by average feeding frequency per 24-hours. Mothers who provided at least a whole day of records (24 hours) were included in the analysis.

6.2.5. Analysis of 3-day infant behaviour dairy

Infant behaviour at 8-weeks was assessed using the 3-day infant behaviour dairy. Mothers were asked to use different symbols that represented “Sleeping”, “Feeding”, “Crying”, “Awake and happy”, “Fussy”, “Colic” behaviours on a 72-hour time scale. Moreover, maternal caring behaviours such as playing, hugging, and changing the nappy could be mentioned on the time scale using “P”, “H”, “C” respectively. However, during the data analysis period, considering the definitions of “crying” “fussy” and “colic” behaviour relied on mother’s understanding and could be varied between mothers, the total time of “crying” “fussy” and “colic” was calculated after removing overlaps; the new variable was re-coded as “distress”. Several mothers also marked “feeding” and “awake & happy” behaviour at the same time based on the concept that their baby was happy and awake during feeding. Therefore, I calculated the total time for “feeding”, “awake & happy” and “playing” on the 3-day dairy with all overlaps removed; the new variable was coded as “Awake (happy)”. Mothers who provided as least a whole day of behaviour records were included into the analysis.

6.2.6. Statistical Analysis

Statistical analysis was conducted in SPSS (version 26.0). Frequencies or percentages were presented for nominal or ordinal data. Normality was checked for continuous data by using Q-Q Plots and histograms. For normally distributed data, the mean±standard deviation (SD) was reported and T-test or ANOVA used for group comparisons; for data with non-normal distribution, the median ± interquartile range (IQR) was reported and non-parametric analysis (Mann-Whitney or Kruskal-Wallis test) was used for group comparisons. Intention-to-treat analyses were carried out for all trial outcomes. Differences between IG and CG mothers at each home visit were compared using independent t-tests for changes in the primary outcomes from 1- to 8-weeks and the values of secondary outcomes (IIFAS, BEBQ, macronutrients composition, energy content, milk intake, and 3-day infant behaviours) at 8-weeks. Pearson correlation was used to examine relationships between variables and Spearman correlation was used to examine dose response effects of the frequency of listening to the relaxation tape with primary outcomes.

To standardise infant weight and length for age and sex, the anthropometric data were converted to standard deviation score (SDS, also known as z-scores) based on the 21st intergrowth data. The conversion can be simply completed by uploading the data in a Microsoft Excel file to the online calculation system: http://intergrowth21.ndog.ox.ac.uk/preterm. The 21st intergrowth study involved participants from eight locations worldwide [246], including the Chinese population.

However, the population in the intergrowth study were preterm infants with a gestational age of 26 to <37 weeks, whilst my study also includes 37-week infants, and this might influence the accuracy of the SDS results. The WHO 2007 standard data, based on the WHO Multicentre Growth Reference Study [247], provided reference values for calculating the SDS of weight and length of term infants. However, the original study did not involve Chinese infants and the mean gestational age was higher than in my study. Considering these factors, I calculated the SDS for infant weight and length using three different methods: 1) all data in my study were converted to SDS by using the 21st intergrowth online calculation system; 2) 34-36 week infants' data were converted to SDS based on the intergrowth data, while 37 week infants data were converted to SDS based on the WHO 2007 standard data for term infants (referred to as Combined Data in later descriptions), by downloading the macro of WHO 2007 data to the STATA for Windows release 16.0 (StataCorp L.P., College Station, Texas); and 3) conditional weight and length gain was calculated based on the standardised residuals for weight/ length in my study population. The standardised residuals were saved from a regression with the measurements at 8-weeks as the dependent variable, and the measurements at 1-week and time between visits as covariates. Then the adjusted mean value was calculated by adjusting infant gender and gestational age to the standardised residuals in order to make the reported value equivalent to the SD scores which also considered gender and gestational age. All three methods used to standardise growth data were used when comparing the changes in infant growth between groups; however, the first method, SDS based on 21st intergrowth data, was the primary outcome when

interpreting the results, because the 21st intergrowth data included infants from Beijing and the gestational age of infants was more similar to my study.

For the evaluation of infant behaviour, independent t-tests were used to identify differences between randomised groups. Pearson correlation was used to test associations between maternal/infant characteristics and infant behaviour.

Apart from the planned statistical analysis as stated in the protocol, exploratory analyses were conducted to test if there were interaction effects for infant gender with the intervention, or gestational week with the intervention. If significant interaction effects were observed, further t-tests or ANOVA were performed for gender or gestation groups on related variables. Moreover, Pearson correlation was used to assess dose-response associations of use of the relaxation tape with primary outcomes and infant behaviours; use of the tape was also compared between mothers of boys and girls. Results of these analyses are outlined in Section 6.6.5. Dose-response effects and 6.6.6 Exploratory analyses of the study outcomes. Full statistical analysis methods are presented in **Table 6.1**. For all results, the significance level was set at $p < 0.05$; however, p values of 0.05-0.1 were regarded as showing a trend/hypothesis-generating for all outcomes.

Table 6.1. Statistical analysis for primary and secondary outcomes of this study.

	Outcomes	Main analysis	Supporting analysis
1	Maternal stress	Independent t-test for changes from 1- to 8-weeks	Independent t-test for 8-weeks value; Interaction analysis using general linear model
2	Maternal anxiety	Independent t-test for changes from 1- to 8-weeks	Independent t-test for 8-weeks value
3	Infant weight	Independent t-test for changes from 1- to 8-weeks	Independent t-test for 8-weeks value; Interaction analysis using general linear model;
4	Infant length	Independent t-test for changes from 1- to 8-weeks	Pearson correlation for dose-response analysis
5	Macronutrients in breast milk	Independent t-test for 8-weeks value	-
6	Milk energy	Independent t-test for 8-weeks value	-
7	Milk intake	Independent t-test for 8-weeks value	-
8	Breastfeeding attitudes	Independent t-test for 8-weeks value	-
9	Infant eating behaviour	Independent t-test for 8-weeks value	-
10	Infant 3-day behaviours	Independent t-test for 8-weeks value	Pearson correlation for dose-response analysis
11	Usage of the relaxation therapy in IG	Descriptive analysis; Pearson correlation between usage and changes in primary outcomes and infant behaviours.	Independent t-test between genders

Notes: If a significant interaction was found for intervention and sex or gestational age, this was further explored using t-test or ANOVA

6.3. Results I: Characteristics of mother-infant dyads

In total, 96 mothers were randomly assigned to IG (n=48) or CG (n=48). All mothers were EBF at 1-week according to the inclusion criteria, while 43.8 % (n=21) IG mothers and 50 % (n=24) CG mothers were EBF until the 8-week home visit. There was no significant difference between groups for the 8-week EBF rate. At the first week home visit, mothers in both groups completed main questionnaires and assessments for primary outcomes and breast milk sample collection. At the 8-week visit, all mothers returned the questionnaire of PSS, BAI, BEBQ, and IIFAS. 92 mothers provided breast milk samples. Three mothers (two IG, one CG) declined to provide a breast milk sample and explained that they had stopped EBF; one mother in the IG left Beijing earlier than expected and could not provide all biological samples, but she posted her questionnaires to me at the 8-week collection. Finally, 48 mother-infant dyads in each group were included in the demographic analysis.

6.3.1. Maternal descriptive characteristics and breastfeeding attitudes

Table 6.2 outlines the descriptive characteristics at baseline for IG and CG mothers. All data were normally distributed. As a result of the stratified randomisation method, no significant differences were found between IG and CG regarding the demographic variables. Mean maternal age was 29.8±3.8 and 29.9±2.9 years in IG and CG respectively. The mean gestational age of infants was 36.1±1.0 weeks in IG and 36.2±0.9 weeks in CG. The maternal attitudes towards breastfeeding were assessed used the IIFAS. The mean

IIFAS score at baseline was 62.1 ± 7.0SD and 62.1± 6.6SD for IG and CG respectively, with no significant difference between groups (p=0.98, CI: -2.7,2.8). However, when checking the individual items in the IIFAS, CG mothers showed more disagreement with the statement of item 2: “Formula-feeding is more convenient than breastfeeding” compared to IG mothers (p=0.045), potentially suggesting greater disagreement with formula feeding in control groups mothers at baseline. No significant differences were found for any other items in IIFAS.

Table 6.2. Baseline characteristics of the included mothers and infants at 1-week according to randomised group.

Descriptive characteristics	Relaxation (n=48)		Control (n=48)		p-value
	N	%	N	%	
Infant's gender					0.84
Male	27	56	25	52	
Female	21	44	23	48	
Gestational age (weeks)					1.00
34 (0/7-6/7)	4	8	3	6	
35 (0/7-6/7)	8	17	7	15	
36 (0/7-6/7)	17	35	18	38	
37 (0/7-6/7)	19	40	20	42	
Location of recruitment					0.98
Northeast Beijing (Centre A)	19	40	20	42	
Central Beijing (Centre B)	6	13	5	10	
Northwest Beijing (Centre C)	11	23	10	21	
South Beijing (Centre D)	12	25	13	27	
Maternal age (years)	30	4	30	3	0.63
Maternal age group (years)					0.72
20-25	5	10	3	6	
26-30	25	54	27	56	
31-35	14	29	15	31	
>35	4	6	3	6	
Marital status					
Married	48	100	48	100	
Educational levels					0.75
School	3	6	1	2	
Certificates/Diploma	12	25	11	23	
Bachelor degree	26	54	30	63	
Postgraduate	7	14	6	13	
Household income (CNY/year)					0.44
<200,000	20	41	20	42	
200,000-300,000	13	27	18	38	

300,000-450,000	6	12	6	13	
>450,000	9	18	4	8	
Birth hospital					0.76
Public hospital	43	90	41	85	
Private hospital	5	10	7	15	
Main maternity care person					0.77
Husband	32	67	28	58	
Parents	10	21	14	29	
In-laws	5	10	4	8	
Confinement lady	1	2	2	4	

Notes: Significance examined by using crosstab. CNY=Chinese Yuan. 1 GBP=8.32 CNY

6.3.2. Early postpartum experience

The early postpartum experiences of mothers were compared between IG and CG and are reported in **Table 6.3.** The experiences during labour and skin-to-skin contact with infant after birth were similar between groups, with no significant differences (all p-values >0.05).

Table 6.3. Early postpartum experiences of mothers in relaxation and control group.

	Control		Relaxation		Total		p-value
	n	%	n	%	n	%	
Mode of delivery							0.49
Vaginal, not induced	17	35	20	42	37	39	
Vaginal, induced	14	29	10	21	24	25	
Planned caesarean	6	13	3	6	9	9	
Unplanned caesarean	11	23	15	31	26	27	
How soon did skin-to-skin contact occur after delivery?							0.54
Directly after birth	13	27	18	38	31	32	
About 15-30 mins after birth	20	42	18	38	38	40	
More than 30 mins after birth	15	31	12	25	27	28	
How long was the skin-to-skin contact after birth?							0.57
Less than 20 mins	27	56	32	67	59	62	
20 mins to 1 hour	15	31	11	23	26	27	
Not within 1 hour after birth	6	12	5	10	11	11	
Instrumental delivery							0.27[a]
Vacuum aspirator	6	67	2	25	8	47	
Forceps	3	33	6	75	9	53	
Medication during labour							0.25
Spinal/Epidural	14	29	20	42	34	35	
Other pain medication	9	19	11	23	20	21	
None medication	25	52	17	35	42	44	
Hospital stays after birth							0.58
Less than 48 hours	6	13	4	8	10	10	
48-72 hours	10	21	14	29	24	25	
More than 72 hours	32	67	30	63	62	65	

Notes: Significance examined by using crosstab (Chi-Square test). [a] The expected count was less than 5 for this variable (minimum expected count is 4), the p-value was thus calculated using Monte Carlo method with 95% confidence interval.

6.4. Results II: Primary Outcomes

6.4.1. Changes in maternal stress and anxiety

Maternal stress was measured using the validated Chinese version of the Perceived Stress Scale (PSS, 14 items). As shown in **Table 6.4**, mothers in IG presented significantly greater reduction of stress from 1-week to 8-weeks compared to CG mothers (p=0.006). In secondary analyses, baseline maternal stress was not significantly different between relaxation and control groups, while at the 8-week home visit, IG mothers presented significantly lower stress compared to CG mothers (p=0.035).

The assessment of maternal anxiety was based on the validated Chinese version of the Beck Anxiety Inventory (BAI). The changes of maternal anxiety from 1-week to 8-weeks were not significantly different between groups; both IG (reduction: 2.9±3.9, 95%CI: 1.71, 4.00, p<0.001) and CG mothers (reduction:1.5±4.3, 95%CI: 0.30, 2.79, p=0.016) presented significant reduction from 1-week to 8-weeks with no difference between groups. In secondary analyses, no significant difference was observed between groups at baseline and at 8-week home visits.

Table 6.4. Comparisons of maternal stress and anxiety between groups.

Groups		**Control**	**Relaxation**			
	n	Mean (SD)	Mean (SD)	p-value	MD	95%CI
Stress [a]						
1-week	96	20.19 (6.8)	20.08 (8.1)	0.94	-0.10	-3.1, 2.9
8-week	96	19.92 (5.9)	17.17 (6.6)	**0.035**	-2.75	-5.3, -0.2
Δ	96	0.27 (5.0)	2.92(4.2)	**0.006**	-2.65	-4.5, -0.8
Anxiety [b]						
1-week	96	8.5 (5.8)	8.4 (6.4)	0.95	-0.08	-2.5, 2.4
8-week	96	6.9 (4.8)	5.5 (5.2)	0.18	-1.40	-3.4.0.6
Δ	96	1.5 (4.3)	2.9 (3.9)	0.12	-1.31	-3.0, 0.4

Notes: [a] assessed by Perceived stress scale (PSS), higher values mean higher stress; [b] assessed by Beck anxiety inventory (BAI), higher values mean higher anxiety; **Δ:** the absolute value of the 8-week value minus the 1-week value; SD=standard deviation; MD=mean difference; CI=confidence interval.

6.4.2. Infant weight and length gain

As shown in **Table 6.5**, infants of relaxation group mothers showed significantly higher weight gain from 1-week to 8-weeks compared to those of control mothers, regardless of the calculation method used (all $p<0.05$). For infant length gain calculated using the 21st Intergrowth data, there was a trend for a decrease in CG with no change in IG.

Table 6.5. Z-score for infant weight, and length from baseline to 8 weeks.

Groups:	Control			Relaxation			T-test			
	n	Mean	SD	n	Mean	SD	*p*	MD	95% CI	
Weight [a]										
1-week	48	0.76	0.8	48	0.71	0.7	0.73	0.05	-0.25	0.35
8-week	48	1.01	0.9	48	1.48	0.8	**0.011**	-0.46	-0.81	-0.11
Weight gain [b]										
A	48	0.26	0.9	48	0.77	0.9	**0.006**	-0.51	-0.88	-0.15
B	48	0.51	0.9	48	0.99	1.0	**0.015**	-0.48	-0.87	-0.10
C	48	-0.27	1.0	48	0.27	0.9	**0.006**	0.55	0.16	0.93
Length [a]										
1-week	48	0.88	0.9	48	0.93	0.7	0.73	-0.06	-0.38	0.26
8-week	48	0.39	1.1	48	0.72	1.0	0.13	-0.33	-0.76	0.10
Length gain [b]										
A	48	-0.37	1.1	48	0.01	1.0	**0.08**	-0.38	-0.81	0.05
B	48	-0.17	0.9	48	0.06	1.0	0.21	-0.24	-0.61	0.14
C	48	-0.14	1.0	48	0.14	1.0	0.17	0.28	-0.12	0.68

Notes: [a] the SDS calculated using 21st intergrowth data for both preterm and term infants; [b] Weight/length gain: the value of the 8-week standard value minus the 1-week standard value; A: changes of SDS calculated using the 21st intergrowth data for both preterm and term infants, B: changes of SDS calculated using the 21st intergrowth data for preterm infants and the WHO term infant data for term infants (Combined Data), C: conditional growth, calculated using the standard residual of 8-weeks infant weight and length and adjusted by gender and gestational age, CI=confidence interval; SD=standard deviation; MD=mean difference.

6.5. Results III: Secondary Outcomes

6.5.1. Macronutrient composition and energy content in breast milk at 8-weeks

Breast milk fat, protein and total carbohydrate at 8-weeks were compared between groups. Overall, no significant differences were observed between IG and CG at the 8-week home visit. When considering the changes in all three macronutrients from 1- to 8-week, a non-significantly greater increase in fat was found in IG mothers compared to CG (0.42 vs. 0.30 g/100ml, p=0.07) (**Table 6.6**).

Table 6.6. Macronutrient composition in breast milk from 1-week to 8-week.

Groups		**Control**			**Relaxation**			**T-test**		
	n	Mean	SD	n	Mean	SD	*p*	MD	95% CI	
Fat(g/100ml)										
1-week	48	2.90	0.40	48	2.92	0.37	0.87	0.01	-0.14	0.17
8-week	47	3.21	0.44	45	3.33	0.41	0.19	0.12	-0.06	0.29
Δ		0.30	0.30		0.42	0.35	**0.07**	0.13	-0.01	0.26
Protein(g/100ml)										
1-week	48	2.06	0.15	48	2.08	0.15	0.41	0.03	-0.04	0.09
8-week	47	0.92	0.07	45	0.94	0.07	0.12	0.02	-0.01	0.05
Δ		-1.14	0.15		-1.14	0.17	0.95	-0.002	-0.07	0.06
CHO(g/100ml)										
1-week	48	6.49	0.39	48	6.44	0.40	0.54	-0.05	-0.21	0.11
8-week	47	6.76	0.26	45	6.85	0.28	0.16	0.08	-0.03	0.19
Δ		0.27	0.48		0.41	0.42	0.13	0.15	-0.04	0.33

Notes: CI=confidence interval; SD=standard deviation; MD=mean difference; CHO= carbohydrate. **Δ:** value of the 8-week value minus the 1-week value.

As shown in **Table 6.7**, there were no significant differences between groups in breast milk energy content at baseline. Similar to the results for fat, a non-significant trend towards a greater energy increase over time was observed in IG mothers compared to CG.

Table 6.7. Energy content (kcal/100ml) in breast milk from 1-week to 8-week home visit.

Groups		**Control**			**Relaxation**		**T-test**			
	n	Mean	SD	n	Mean	SD	*p*	MD	95% CI	
1-week	48	61.17	5.45	48	61.19	5.19	0.98	0.02	-2.13	2.17
8-week	47	60.58	4.74	45	62.08	4.57	0.13	1.50	-0.43	3.43
Δ		-0.72	4.39		1.03	4.33	**0.06**	1.76	-0.06	3.56

Notes: CI=confidence interval; SD=standard deviation; MD=mean difference; **Δ:** value of the 8-week value minus the 1-week value.

6.5.2. Breast milk intake and infant appetite

A total of 36 mothers returned the 48-hour test weighing form. Ten mothers recorded weight after all feeds during the 48 hours; 17 mothers recorded all their feeds during the first 24-hours and some feeds in the second 24 hours. Milk intake of these 27 mothers (IG=13, CG=14) was estimated by using the formula given in Section 6.3.4. The remaining nine mothers were excluded from the analysis due to the limited records obtained. For the 27 included mothers, one in the CG and two in the IG were mixed feeding at 8-weeks and the volume of formula was included in the total milk intake; there was no significant difference between groups regarding the feeding methods.

After adjusting for 5% IWL, the mean value of the estimated milk intake was 558±42g in CG mothers and 559±36g in IG mothers with no significant difference between groups.

Infant appetite was assessed at 1- and 8-week home visits using the Baby Eating Behaviour Questionnaire (BEBQ). The mean general appetite score increased from 1-8 weeks (4.33 to 4.48) in the IG and slightly decreased (from 4.38 to 4.31) in the CG, but the changes were not significant between groups (**Table 6.8**). Moreover, the change in values were not significantly different between groups for enjoyment of food, food responsiveness, slowness in eating, and satiety responsiveness.

Table 6.8. Baby eating behaviour at 1-week to 8-week home visit.

	Control			Relaxation			T-test			
Variables	n	Mean	SD	n	Mean	SD	p-value	MD	95% CI	
1-week										
Enjoyment of food	48	4.1	0.6	48	4.2	0.5	0.926	0.01	-0.2	0.2
Food responsiveness	48	3.1	0.6	48	3.3	0.7	0.206	0.17	-0.2	0.4
Slowness in eating	48	2.8	0.7	47	2.9	0.7	0.429	0.12	-0.2	0.4
Satiety responsiveness	48	2.5	0.7	48	2.6	0.8	0.327	0.15	-0.2	0.5
General appetite	48	4.4	0.7	48	4.3	0.7	0.771	-0.04	-0.3	0.2
8-weeks										
Enjoyment of food	48	4.1	0.6	48	4.2	0.4	0.465	0.07	-0.1	0.3
Food responsiveness	48	3.2	0.6	48	3.2	0.6	0.743	0.04	-0.2	0.3
Slowness in eating	48	2.5	0.6	47	2.7	0.7	0.188	0.18	-0.1	0.4
Satiety responsiveness	48	2.6	0.8	48	2.4	0.6	0.428	0.43	-0.4	0.2
General appetite	48	4.3	0.6	48	4.5	0.5	0.167	0.17	-0.1	0.4

Notes: CI=confidence interval; SD=standard deviation; MD=mean difference

6.5.3. Maternal attitudes towards breastfeeding at 8-weeks

The maternal attitudes toward breastfeeding were assessed by IIFAS. **Table 6.9** shows the mean score of each individual item of the IIFAS and the total IIFAS score for relaxation and control groups at 8-weeks. No significant difference was found in total IIFAS score between groups. Both groups had a generally positive perception towards breastfeeding at 8-weeks. When checking the individual items in the IIFAS, the IG mothers showed significantly higher disagreement on item 8: "Women should not breast-feed in public places such as restaurants" ($p=0.026$) and significantly higher agreement on item 9 "Babies fed breast milk are healthier than babies who are fed formula" ($p=0.002$) compared to CG mothers. No significant differences were found for any other items.

Table 6.9. Comparison of maternal attitudes towards breastfeeding at 8-week postpartum between relaxation and control group.

Items	Relaxation (n = 48)		Control (n=48)		t-test for Equality of Means		
	Mean	SD	Mean	SD	p-value	95% C.I.	
1. The nutritional benefits of breast milk last only until the baby is weaned from breast milk. ǂ	3.19	1.48	3.35	1.30	0.56	-0.73	0.40
2. Formula-feeding is more convenient than breastfeeding. ǂ	3.60	1.18	3.73	0.96	0.57	-0.56	0.31
3. Breastfeeding increases mother-infant bonding.	4.56	0.71	4.52	0.83	0.79	-0.27	0.35
4. Breast milk is lacking in iron. ǂ	3.63	0.89	3.73	1.00	0.59	-0.49	0.28
5. Formula-fed babies are more likely to be overfed than breast-fed babies.	3.48	1.20	3.69	0.97	0.35	-0.65	0.24
6. Formula-feeding is the better choice if a mother plans to work outside home. ǂ	3.02	1.10	2.98	1.04	0.85	-0.39	0.48
7. Mothers who formula-feed miss one of the great joys of motherhood.	3.94	1.10	3.96	1.03	0.92	-0.45	0.41
8. Women should not breast-feed in public places such as restaurants. ǂ	3.65	1.23	3.08	1.20	**0.026**	0.07	1.06
9. Babies fed breast milk are healthier than babies who are fed formula.	4.21	0.94	3.63	0.82	**0.002**	0.23	0.94
10. Breast-fed babies are more likely to be overfed than formula-fed babies. ǂ	3.65	1.12	3.77	0.83	0.54	-0.53	0.28
11. Fathers feel left out if a mother breast-feeds. ǂ	3.52	1.05	3.52	0.99	1.00	-0.41	0.41
12. Breast milk is the ideal food for babies.	4.50	0.68	4.54	0.71	0.77	-0.33	0.24

13. Breast milk is more easily digested than formula.	4.48	0.74	4.3	0.86	0.38	-0.18	0.47
14. Formula milk is as healthy for an infant as breast milk. ǂ	2.75	1.12	2.85	0.92	0.62	-0.52	0.31
15. Breastfeeding is more convenient than formula feeding.	3.67	1.14	4.04	0.87	0.07	-0.79	0.04
16. Breast milk is less expensive than formula.	4.23	1.06	3.96	1.07	0.22	-0.16	0.70
17. A mother who occasionally drinks alcohol should not breast-feed her baby. ǂ	2.23	1.29	2.19	1.30	0.88	-0.48	0.57
Total scores of IIFAS	62.29	7.28	61.88	6.49	0.77	-2.38	3.21

* p-value < 0.05; Higher score means more positive attitudes toward breastfeeding. ǂ Unfavourable to breastfeeding (reversed score applied). CI=confidence interval.

6.5.4. Infant behaviour: results of the 3-day infant behaviour diary

Overall, 58 of the 96 mothers returned the dairy (compliance rate 60.4%), including 28 mothers in CG and 30 in IG. For the 58 mothers, 47 recorded the infant behaviour for three whole days, two mothers completed two days and a half, three mothers completed one day. Moreover, six of the 58 mothers only completed the day-time behaviour of the infants with no night-time behaviours recorded; of these, four mothers completed three days of daytime behaviour, one mother completed two days and one completed only one day. To calculate the whole day behaviours of the infants more accurately, the six mothers who did not include night-time infant behaviours were excluded from the analysis. Moreover, one mother only provided very scribbled records of sleeping behaviours for the first day, which was difficult to read, and other behaviours were not mentioned; thus, this mother was excluded, leaving a total of 51 mothers for data analysis. However, some mothers did not provide any record on “crying” “fussy” and “colic”, meaning the “distress” behaviour recorded was zero. This does not seem plausible for a healthy infant; hence two analyses were conducted; one included all 51 mothers using the value 0 for mothers who did not record distress behaviours; the other included 41 mothers who reported at least one of the “distress” behaviours. Results of both analyses are shown in the Section 6.6.4.

All behaviours were normally distributed. There were no significant differences in socio-demographic characteristics of mothers between those who did and did not complete

the diary (all p>0.05). The completion rate was not significantly different between IG and CG mothers (IG=26 vs. CG=25, p>0.05). The mean values of infant behaviours per day are outlined in **Table 6.10**.

Table 6.10. Total duration (minutes) of infant behaviour during a day at 8-weeks.

	Control			Relaxation			T-test			
Variables	n	Mean	SD	N	Mean	SD	Sig	MD	95% CI	
Sleeping	25	794	150	26	827	141	0.43	-32.7	-114.5	49.2
Awake (happy)	25	340	127	26	359	114	0.57	-19.4	-87.2	48.5
Distress	25	66	62	26	55	64	0.53	11.0	-24.2	46.3
Distress (record)*	20	82	58	21	68	64	0.46	14.5	-24.3	53.2

Notes: MD=mean difference. *Distress (record) = results including mothers who reported at least one of the "crying" "fussing" and "colic" behaviours while Distress = results including all 51 mothers and assuming the value was 0 if the mother did not provide any record of "crying" "fussing" and "colic" behaviours.

Overall, no significant differences were found between IG and CG mothers for infants' time spent sleeping, awake (happy) and distressed per day. Pearson correlation results showed that lower maternal stress at 8-weeks was significantly correlated with longer awake (happy) duration per day (r=-0.279, p=0.047) and more frequent awake & happy behaviours (r= -0.343, p=0.032). Other assessed behaviours were not correlated with maternal perceived stress at 8-weeks (all p>0.05).

6.5.5. Dose-response effects

For the use of the relaxation meditation tape in IG mothers, a diary was given to mothers to record the duration of listening after use. All mothers in the IG were encouraged to

listen to the therapy at least once a day before breastfeeding. Regular telephone interviews were conducted at 1-month postpartum and the nurse gently reminded/encouraged the mother about the benefits of continuing to use the tape. Overall, 41 of the 48 IG mothers returned the recording diary (completion rate 85%). For the remaining seven mothers who did not return the diary, three said they did listen to the relaxation tape but felt it too cumbersome to record this. Three expressed that they did not feel it worked well and gave up using it after 3-5 days. One stated that she lost her record form and could not remember the exact days she listened to the recording. Hence, data from 41 mothers was used for the dose-response analysis.

The data for use of the therapy are shown in **Table 6.11**. The majority of mothers (83%) had listened to the tape for more than 20 days. 71% mothers had listened to the tape on more than 28 days, which accounted for half of the follow-up period. Given these figures, the compliance with the intervention was reasonably good. Most mothers listened to the tape only once a day, while four mothers listened more than once on some of the days. The reported listening duration varied among individual participants, ranging from 1 to 30 minutes for an individual session. No significant differences were found in the total duration or average use of the relaxation therapy between mothers of girls and boys, however mothers of girls reported significantly more days of listening to the therapy compared to mothers of boys (45±10 vs. 34±16 days, p=0.015) (**Figure 6.2**).

Table 6.11. Descriptive statistics for the frequency of listening to the therapy (minutes)

	N	Total duration		Total days		Average usage per day	
		Mean	SD	Mean	SD	Mean	SD
Girl	19	481	133	45	10	11	1
Boy	22	382	228	34	16	10	2
Total	41	428	195	39	15	10	2

Note: SD =standard deviation

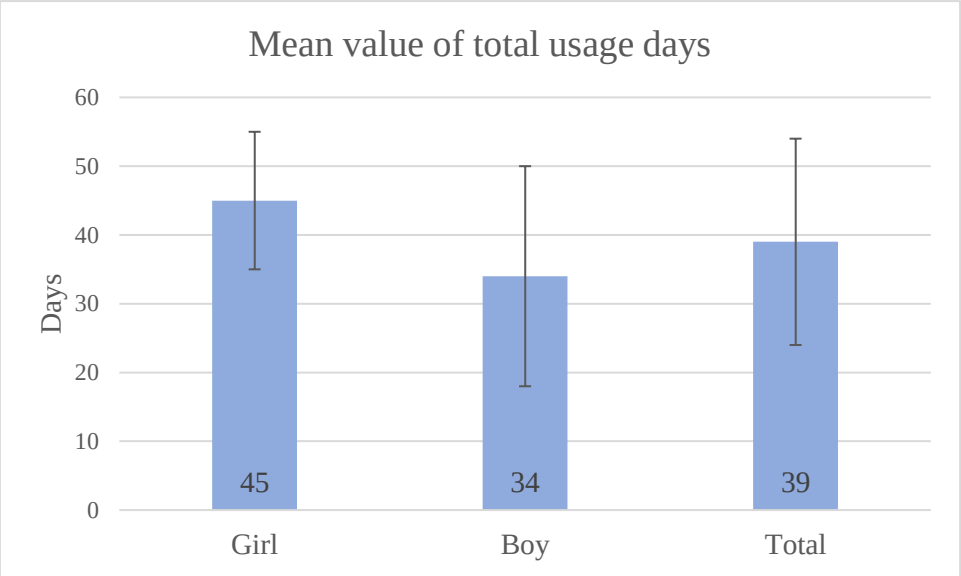


Figure 6.2. Mean of the total usage days of the relaxation therapy.

Notes: Y-axis values shows the total mothers and mothers of girls/boys in intervention group who provided data on their usage of the relaxation tape. X-axis shows the mean value of the total usage days of the relaxation tape.

Moreover, **Table 6.12** shows the correlations between the use of the relaxation tape (mean/total duration, days of usage) and maternal stress, anxiety, infant weight and length gain, and infants' sleeping, crying, and awake/happy behaviours. Greater use of the relaxation tape was associated with greater reduction in maternal stress, while a greater number of days of use was associated with higher infant weight at 8-weeks (assessed using the Combined Data SDS), with a non-significant trend for total minutes of use during the study period. No significant correlations were found for maternal anxiety and infant sleeping, crying, and awake/happy behaviours.

Table 6.12. Pearson correlation among the usage of intervention and infant outcomes.

		Total duration (minutes)		Total usage (days)		Duration/day (minutes)	
	N	R	p	r	P	r	p
Maternal Stress							
At 8-weeks	41	0.07	0.650	0.15	0.339	-0.209	0.190
Δ	41	**-0.55**	**0.000**	**-0.58**	**0.000**	**-0.435**	**0.005**
Maternal Anxiety							
At 8-weeks	41	0.16	0.334	0.24	0.125	-0.068	0.673
Δ	41	-0.02	0.913	0.02	0.894	-0.280	0.076
Infant Weight A							
At 8 weeks	41	0.24	0.124	0.25	0.115	0.175	0.274
Δ	41	0.13	0.437	0.06	0.735	0.250	0.115
Infant Weight B							
At 8 weeks	41	0.29	0.065	**0.32**	**0.044**	0.196	0.219
Δ	41	0.11	0.505	0.03	0.855	0.233	0.143
Infant Length A							
At 8 weeks	41	0.15	0.360	0.19	0.245	0.021	0.898
Δ	41	0.06	0.702	0.03	0.857	0.112	0.485
Infant Length B							
At 8 weeks	41	0.20	0.214	0.25	0.121	0.072	0.655
Δ	41	0.22	0.175	0.25	0.122	0.001	0.996
Infant Behaviour							
Sleeping Duration	22	0.06	0.783	-0.07	0.759	0.385	0.077
Crying Duration	11	-0.35	0.291	-0.16	0.637	-0.353	0.287
Awake/happy Duration	15	-0.08	0.789	-0.11	0.695	-0.195	0.487

Notes: **Δ:** value of the 8-week value minus the 1-week value; A: SDS calculated using 21st intergrowth data; B: SDS calculated using Combined Data.

6.5.6. Exploratory analyses

Based on the significant results observed regarding the reduction of maternal stress and increase of infant weight in the IG, general linear models were used to test for interactions between the intervention and infant gender or gestational age in relation to the changes in maternal stress and infant weight. Considering that the number of infants with gestational age of 37 weeks accounted for nearly half of all infants, those with gestational age of 34-36 weeks were merged and coded as “late preterm” whilst 37-week infants were coded as “early term” in the following analysis. Results are shown in **Table 6.13**.

Table 6.13. Results of the interaction analysis conducted by the general linear model.

		B	P-value	95%CI	
Maternal stress changes					
Relaxation	Boy (ref)	-	-	-	-
	Girl	-1.33	0.49	-5.18	2.52
	Early term (ref)	-	-	-	-
	Late preterm	-0.74	0.71	-4.65	3.17
Infant weight gain (Intergrowth)					
Relaxation	Boy (ref)	-	-	-	-
	Girl	**0.54**	**0.039**	**0.03**	**1.05**
	Early term (ref)	-	-	-	-
	Late preterm	0.11	0.78	-0.65	0.87
Infant weight gain (Combined Data)					
Relaxation	Boy (ref)	-	-	-	-
	Girl	**0.77**	**0.038**	**0.04**	**1.50**
	Early term (ref)	-	-	-	-
	Late preterm	0.11	0.77	-0.64	0.87
Conditional weight gain					
Relaxation	Boy (ref)	-	-	-	-
	Girl	0.55	0.17	-0.24	1.34
	Early term (ref)	-	-	-	-
	Late preterm	0.16	0.69	-0.65	0.97

Notes: CI=confidence interval; ref=reference value.

There was no significant interaction between randomisation group and infant gender or gestational age on changes in maternal stress. However, the relaxation intervention showed significantly greater effects on weight gain in girls than in boys, when SDS were calculated using 21st intergrowth data or Combined Data (**Figure 6.3** and **Figure 6.4**). No significant interaction effect was found when using the conditional weight gain for the same assessment (**Figure 6.5**).

In addition, it should be mentioned that when assessing the interaction effects of gender and relaxation intervention on infant growth using the actual weight gain instead of the SDS, there was no significant effect. There was also no interaction between gestational age and relaxation therapy on infant growth.

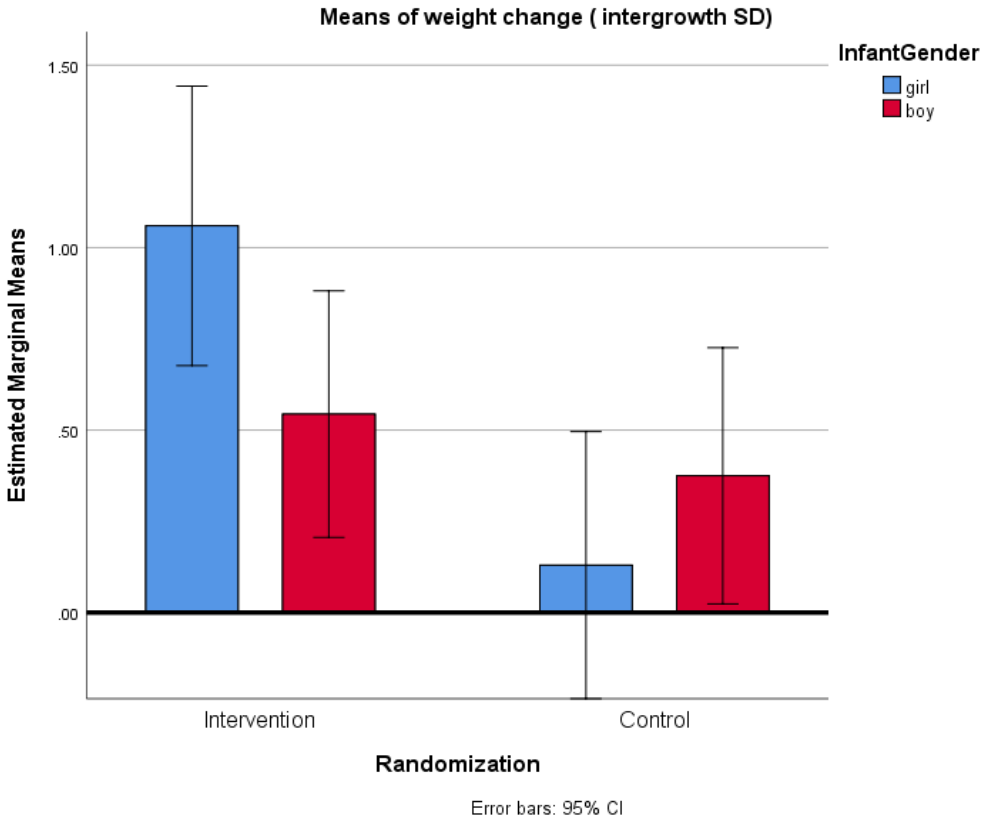


Figure 6.3. Gender differences in weight gain using 21st intergrowth SDS.
Notes: intergrowth SD=SD score calculated using the 21st intergrowth data; CI=confidence interval

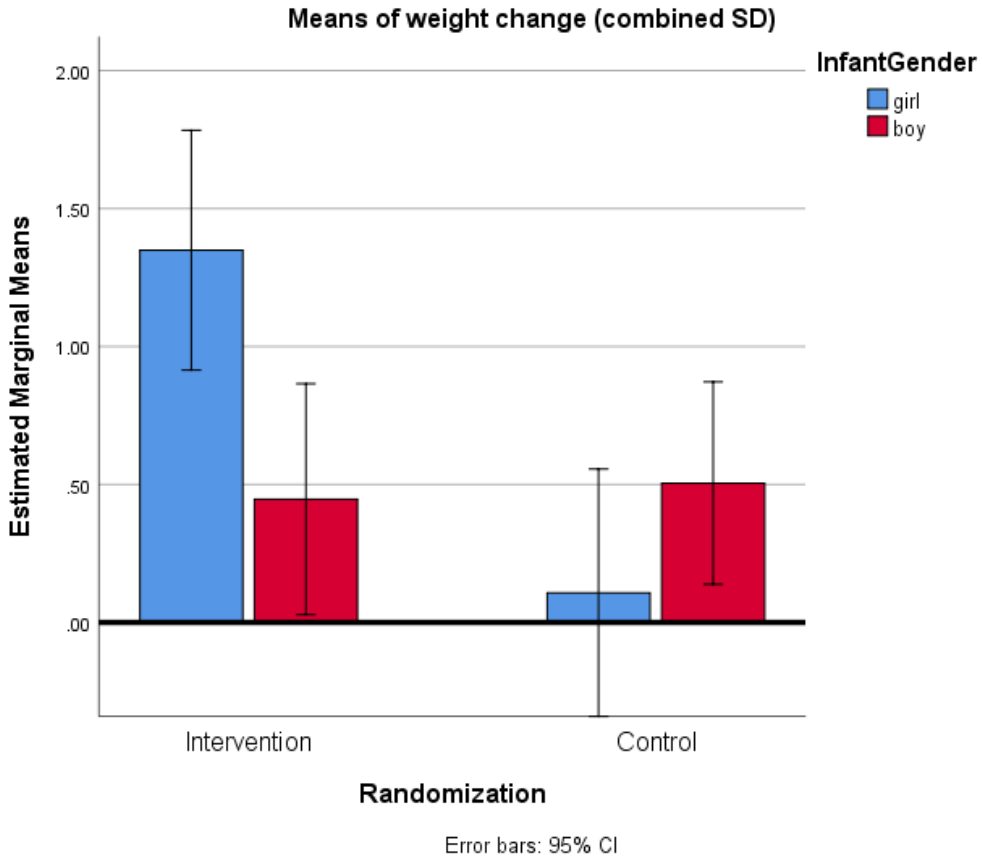


Figure 6.4. Gender differences in weight gain using combined intergrowth and WHO SDS.

Notes: combined SD= SD score calculated using combined WHO term data (for 37 weeks infants) and 21st intergrowth data (for 34-36 weeks infants); CI= confidence interval

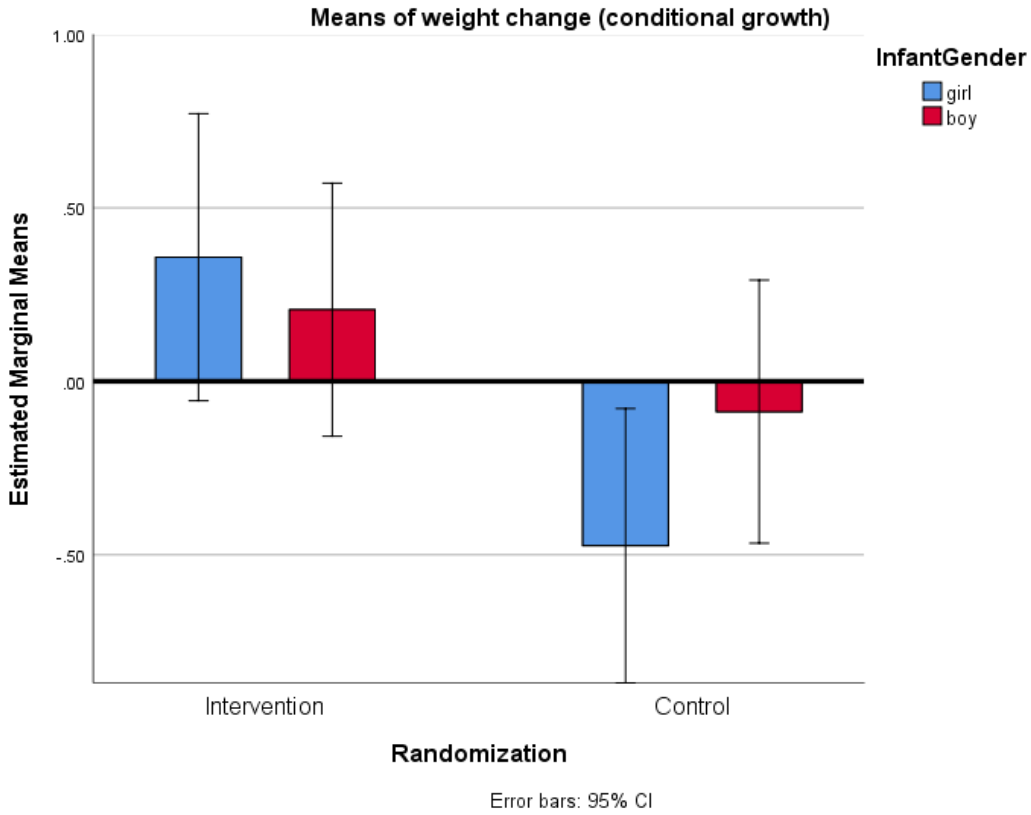


Figure 6.5. Gender differences in conditional weight gain.
Notes: CI= confidence interval

To further investigate the interaction between intervention and infant gender on weight gain I conducted independent t-tests to compare the breastmilk macronutrient and energy content changes between infant genders within both IG and CG. While no significant gender differences were observed in the CG, a significant increase in breastmilk energy from 1- to 8-weeks was observed in mothers of female infants, compared to male infants in IG (2.5±4.8 vs. -0.1±3.6 kcal/100ml, p=0.046; **Figure 6.6**). A non-significant trend of fat increase was also observed in mothers of female infants in IG (0.53±0.41 g/100ml) compared to boys (0.32±0.42 g/100ml), p=0.073; **Figure 6.7**).

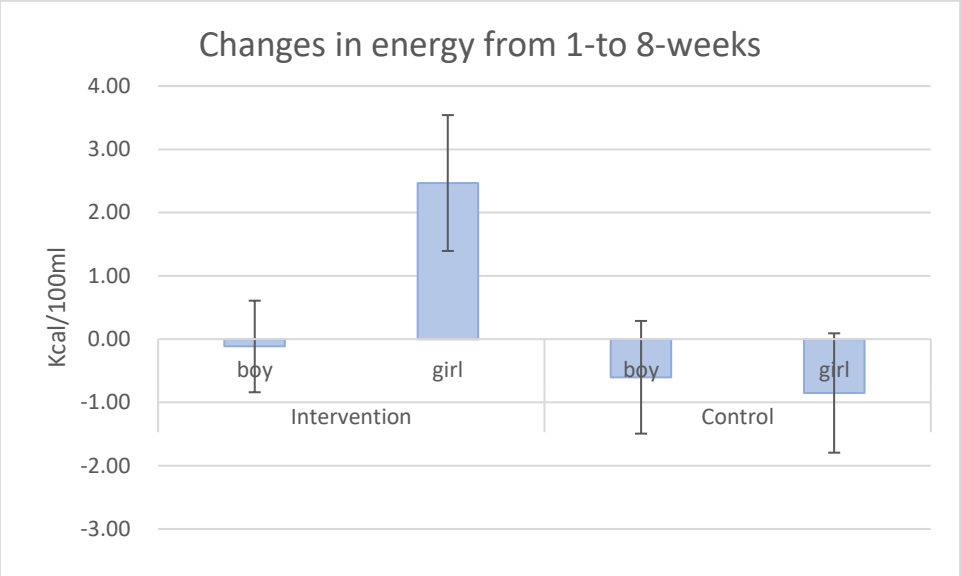


Figure 6.6. Comparisons between genders in relaxation and control groups regarding the changes in breast milk energy from 1- to 8-weeks (Error bars: standard error).

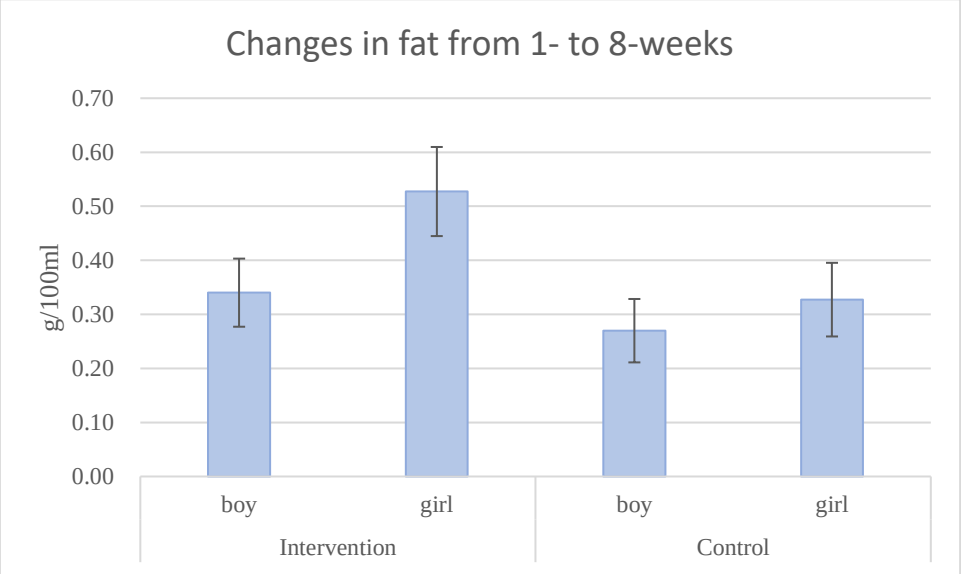


Figure 6.7. Comparisons between genders in relaxation and control groups regarding the changes in breast milk fat from 1- to 8-weeks (Error bars: standard error).

6.6. Discussion

6.6.1. Summary of the findings

- Baseline Results:
  a) No significant differences were found either in maternal socio-demographic factors or early postpartum experience between IG and CG.
  b) Both group of mothers presented a positive attitude toward breastfeeding with no significant group differences.
- Primary Outcomes:
  a) A significant reduction of maternal postpartum stress was observed in IG mothers; greater use of the relaxation tape was associated with significantly greater reduction in maternal stress, whilst no significant changes were found in maternal anxiety between groups.
  b) Compared to CG infants, IG infants showed significantly greater weight gain and a non-significant trend for greater length gain from 1-to 8-weeks. A greater number of days of use of the intervention was associated with significantly higher infant weight at 8-weeks.
- Secondary Outcomes:
  a) No significant differences were observed for fat, carbohydrate, protein, and energy content between groups at the 8-week home visit, although a non-significant trend of increase was observed for breast milk fat and energy content in IG mothers, compared to the CG.

b) Only 27 mothers were included into the milk intake analysis with no significant difference between groups.

c) No significant differences were found in either total BEBQ scores or any categories of the BEBQ.

d) No significant differences were found in maternal breastfeeding attitudes, although the IG mothers presented significantly more positive attitudes in item 8 “Women should not breast-feed in public places such as restaurants” and item 9 “Babies fed breast milk are healthier than babies who are fed formula” of the IIFAS.

e) No significant differences were found in total duration of any assessed infant behaviours between IG and CG; reduced maternal stress was significantly associated with longer awake/happy duration and more frequent awake (happy) periods.

f) A significant interaction effect was observed for the intervention and infant gender on weight gain; the effect of the intervention on weight gain was significantly greater in girls than in boys. Moreover, significantly more milk energy and a non-significant trend for higher milk fat were observed in mothers of girls in IG.

g) Mothers of girls reported significantly more days of listening to the therapy compared to mothers of boys

6.6.2. Maternal psychological changes

Consistent with the present study, the effect of relaxation intervention on reducing maternal stress has been previously demonstrated in several experimental studies [120, 125, 248-251]. However, the sample size in most studies was small (20-40) [120, 248]. Moreover, some experimental studies used a pre/post-test design, which could only show an acute effect of the relaxation intervention, leaving the long-term and dose-response effects of intervention under-investigated [249, 251]. The present study had a longer follow-up period of 8 weeks. The significant reduction in PSS score in IG mothers suggests that listening to the relaxation tape from 1-week to 8-weeks may have effects on reducing postpartum stress. More convincingly, the dose response analysis suggests that the longer the duration and/or the more frequently the tape was used, the greater reduction of maternal stress observed. Consistently, the MOM study reported similar results with significant effects of relaxation therapy on reducing maternal stress at 6- and 12-weeks, including a dose-response effect.

However, it should be mentioned that the assessment tool used to record maternal stress in the present study was a self-reported questionnaire, which could introduce reporting bias. Although both groups of mothers were unaware of the randomisation to intervention or control groups, IG mothers might either expect to feel less stressed after using the relaxation tape or believe that they are expected to report this by the researcher. However, in the pilot study performed prior to this RCT, the relaxation tape showed significant effects on reducing heart rate, SBP, DBP, and in increasing fingertip

temperature which, as discussed in Chapter 3, reflect objective relaxation responses [118]. Similarly, an experimental study conducted in 129 Chinese mothers with preterm labour showed an acute effect of a 13-minute relaxation programme on reducing visual analogue scale stress scores and increasing finger temperatures [251]. Hence, both physiological and psychological outcomes have shown acute effects of relaxation therapy on reducing maternal stress and the effects of relaxation therapy on reducing maternal stress are plausible. However, further objective measurements on the long-term effects of relaxation therapy could be included in future studies.

Consistent with the MOM study, no significant changes in maternal anxiety were found between groups [124]. The authors of the MOM study suggested that the effects of intervention on this outcome might be reduced due to the small sample size and the relatively low mother-infant conflict (tension) at baseline, considering the mothers had a healthy term labour. Compared to the MOM study, the tension between mother and infants might be higher in my study population following late preterm and early term delivery. The sample size in my study was also larger, but I still found no effect of the relaxation treatment on maternal anxiety.

An RCT conducted by Dabas et al. [125] including 60 preterm delivered mothers showed significant reduction in both maternal stress and anxiety following a relaxation intervention. However, it should be mentioned that the intervention duration was short (10 days), and the long-term effects were not investigated. Another RCT conducted by Varisxoglu et al.[120] investigated the effects of music therapy on the reduction of

postnatal anxiety in mothers of premature infants. Results showed a significant reduction of maternal anxiety in IG mothers, which was inconsistent with the present study. However, the duration of the intervention was only four days hence only the acute effects of the therapy were shown. Moreover, the gestational age of infants was 26-33 weeks in Dabas et al and 28-34 weeks in Varisxoglu et al., so their mothers may have experienced greater anxiety compared to the present study population, potentially increasing the chance of detecting significant differences after the intervention. Additionally, the assessment tool used to assess anxiety can be an influencing factor. Dabas et al. and Varisxoglu et al. used the Perinatal Anxiety Screening Scale (PASS) and State Trait Anxiety Inventory-State (STAI), respectively, and the use of different assessment tools can reduce the comparability to some extent. Further, although the Chinese version of BAI had been demonstrated to have a reasonable validity and reliability[252], such studies in China were mostly conducted in patients with chronic diseases or older people [252, 253] who may be more vulnerable and suffering from severe health issues compared to the postpartum women in my study. Hence, the validity and reliability of BAI in postpartum women needs to be further confirmed.

Another explanation for the effects of relaxation therapy on reducing maternal stress but not anxiety in my study could be that, compared to stress, which might commonly be reported in otherwise healthy adults, anxiety may be less common and more often associated with other mental health issues. When comparing the content between the PSS and BAI it is notable that some dramatic descriptions are provided in the BAI, such

as “difficulty in breathing” and “fear of dying”. Such items might not make sense to the mothers, resulting in a lower BAI score in both 1-week and 8-weeks assessments. Also, the relaxation intervention might focus more on helping people to relax rather than treating more severe symptoms of anxiety.

6.6.3. Infant growth

For the measurements of infant growth, SDS were calculated to standardize infant weight and length for infant age and sex, and conditional weight and length gain were also calculated to adjust later measurements for the baseline weight and length and the duration between data collection. All methods demonstrated significantly higher weight gain in IG infants compared to CG infants. This finding reflects a positive effect of relaxation therapy on infant weight gain, which was consistent with the MOM study [124].

Interestingly, the interaction analysis suggested a greater effect of the intervention on weight gain in girls than boys. Whilst the absolute amount of weight gain in the IG group was not significantly different between boys and girls, the weight gain expressed in SDs was greater in girls than in boys, which suggests the weight gain is greater than expected in girls (when compared to the reference population of girls) but not for boys. Moreover, there was a significant increase of milk energy content in IG mothers with female infants whilst in mothers of boys, the energy content slightly decreased. Besides, there was a trend for increased milk fat content in IG mothers with girls. It is possible that the

increased breast milk fat and energy content contributed to the greater relative weight gain in IG girls compared to boys.

The increase in weight SDS in CG girls was less than that in CG boys, which is consistent with published papers where the growth rate in the first 6 months was faster in male infants than females [254, 255]. Trivers-Willard's theory [256] predicted a sex-biased investment in offspring among mammals; based on this, male infants, who are heavier at birth and grow faster than females, incur greater short-term costs, thus mothers may produce sex-biased milk to meet their requirements. Animal studies in rhesus macaques and calves indicated that mothers of sons produced greater yields of milk, milk protein, fat and lactose than mothers of daughters [66, 257]. A cross-sectional study further confirmed these findings in humans; analysis of milk samples from 25 EBF mothers at 2-5 months after delivery showed that mothers of male infants produced milk that had 25% greater energy content than mothers of female infants ($P < 0.001$)[258]. Although the sample size was small, the author suggested that the increased energy content of milk produced by mothers of male infants may result from the greater energy requirements of male infants who then presumably demand more feeds or suck more vigorously to upregulate milk production.

However, it is widely recognised that boys are more vulnerable than girls, with excess male morbidity and mortality universally reported [259]. A systematic review and meta-analysis showed that boys had higher odds of being underweight than girls in children under five years old (pooled OR=1.14, 95% CI: 1.02-1.26) [260]. Hack et al. [261] reported

a higher increase in weight SDS from 1 to 8 months in preterm girls. However, the author suggested that this finding might relate to the lower morbidity shown by girls during neonatal period. Another cohort study investigating if maternal feeding choice at 4 months of age influenced subsequent growth suggested that male infants who were breast-fed grew more slowly in infancy than those who were formula-fed, whilst no significant difference was observed in breast-fed versus formula-fed girls [262]. Nonetheless, the author did not suggest potential mechanisms for these findings.

Gender differences in undernutrition status might be more significant in 'stressful' situations. For example, in several studies conducted in East Africa, in resource-poor settings, indicated that wasting, stunting and underweight are commonly experienced by male infants/children [263, 264]. A cross-sectional study in Uganda including 723 mother-infant pairs showed that the length SDS was significantly lower in boys than girls (-0.65 vs. -0.37, p=0.03) and stunting was more prevalent among boys than girls (58.7% vs. 41.3%) [265]. However, only the surviving infants were included in the study, and that in itself entails an inbuilt selection bias.

Consistent with these findings, the results in my study showed greater weight gain in female infants, suggesting that the relaxation intervention might alter maternal investment in girls more than boys. However, it should be noted that compared to mothers of boys in the IG, mothers of girls spent significantly more days listening to the relaxation tape, hence the beneficial effects on greater weight gain in girls might reflect greater exposure to the intervention. Therefore, mothers of girls might be more relaxed

and the let-down reflex may have been more efficient, leading to a higher hind milk intake [18, 266]. Given this, the significantly higher energy content and the trend of higher increase in breast milk fat, which may contribute to the greater weight gain in girls in the IG, can be explained by the greater exposure to the intervention. While it can be hypothesised that maternal compliance with the use of relaxation tape was influenced by gender-specific infant behaviours, no significant difference was found in any of the infant behaviours between genders. Another potential factor that could explain different usage of the relaxation tape between genders might be the traditional concept that sons have a higher status than daughters in Chinese culture because they can carry on the family name [267, 268]. A qualitative study in China reported the conflicts between new mothers and their mothers-in-law due to the fact that they gave birth to a baby girl: one woman was particularly stressed and claimed that her mother-in-law did not like her daughter and put a huge pressure on her [269]. Another study showed that not having a male baby could cause extreme stress among new parents in mainland China, which was associated with the fact that it is not uncommon for women to undergo sex-selection abortion after ultrasonography[270]. As a result, mothers of girls might experience more stress [271] and this could result in more frequent use of the relaxation therapy. On the other hand, it can be suggested that mothers of female infants benefited more from the relaxation therapy, so that they saved more energy and could invest more in the infant; consistent with the trend of higher increase in breast milk fat and energy content. However, as the total usage days of the intervention was significantly different between genders, it could not be concluded that there was greater

investment in female infants by their mothers. To identify if the gender differences in growth resulted from altered investment by mothers or was due to different exposure to the intervention it would be helpful to collect information regarding why and when a mother decided to use the relaxation tape, or to standardise the use of the intervention by mothers of male and female infants, although this might be difficult in practice.

A non-significant trend for higher length gain was observed in IG infants compared to CG infants in my study. The non-significant trend for length gain might partly reflect the quality of the measurements; although standard training was conducted regularly throughout the study period, the measurement of length was still more subjective compared to the weight which was measured using digital scales. It was also subject to inter-observer error given the fact that measurements were made by several different nurses. Although there could be a potential risk of detection bias if the research nurse overestimated the length of IG infants, this was unlikely in my study, since the research nurses had no particular interest or investment in the results of the study. Hence, I consider the risk of detection bias to be low.

The present study used different reference data when calculating the SDS. Since the eligible infants were born from 34 0/7 to 37 6/7 gestational weeks, it is difficult to say whether the 21st intergrowth data (26 0/7 – 36 6/7 weeks) or the Combined Data (21st intergrowth data for calculation of 34 0/6 -36 6/7 weeks infants, WHO term data for calculation of 37 0/7 -37 6/7 weeks infants in the present study) are more reasonable. Whilst the limitation of using 21st intergrowth data for early term infants may lead to

overestimated SDSs considering the reference data were from preterm infants, using the WHO term infant data can result in underestimation of the SDS, since the mean gestational weeks in the WHO dataset were higher than 37 weeks. Moreover, compared to the intergrowth study which included mothers from Beijing, the WHO data did not include a Chinese population. Hence, errors might result from differences in growth between ethnic groups. Conditional growth was thus applied to triple check the changes regarding weight and length gain. Significantly greater weight gain was shown using all three methods, increasing confidence in the finding.

It is relevant to consider whether the significantly greater weight gain in IG infants should be considered more optimal than that in the CG. Although studies showed that rapid growth in infancy may protect preterm infants from cognitive impairment, it may also be associated with increased risk of childhood obesity or cardiometabolic risk in later life [272]; this suggests that there could be a trade-off in preterm babies whereby providing enhanced nutrition to prevent postpartum growth faltering results in better cognitive outcomes, yet at the same time, the accelerated weight gain can increase the risk of later metabolic and cardiovascular disease [272]. However, data from existing studies are mostly focused on preterm infants under 32 weeks GA, rather than LP and ET as in my study. It is unclear if the growth patterns and the consequences of rapid growth would be different specifically in LP and ET. Besides, the SDS in the present study were not much above the median, especially the SDS calculated by intergrowth data, hence the increased weight gain might not be regarded as “rapid growth” but rather

'optimal'. Results using conditional growth showed that compared to the 1-week weight and length data, the IG infants gained more weight than expected while CG infants gained less, hence, it was difficult to draw conclusions regarding the potential risk of increased weight gain in IG. Moreover, in addition to body weight, other aspects of growth such as lean body mass should also be taken into account when assessing the quality of postnatal growth, and this was not measured in the present study. Hence more data and a longer follow-up period are ideally required to evaluate if the increased weight gain can be seen as more optimal growth in these LP and ET infants.

The following sections discuss other study findings and consider the increased infant growth observed in the IG from the perspectives of breast milk and infant behaviours.

6.6.4. Breast milk outcomes

As shown in the secondary results, a non-significant increase in breastmilk fat and energy content were observed in IG mothers. The non-significant results may be due to two reasons. Firstly, only fore milk was collected in this study and the energy content was accordingly calculated using the nutrient value from the fore milk sample. However, studies have shown that the fat content in the hind milk could be approximately two- to threefold that of foremilk, and hindmilk has approximately 25–35 kcal/100 ml more energy on average than foremilk [273-275]. Hence, it is possible that the fat content in the hind milk of IG mothers might be even higher than that in the CG; besides, the let-down reflex improved by the relaxation intervention might also lead to a greater

difference between groups, which could explain the observed greater infant growth. Secondly, there were some methodology issues during the data collection process, even though I attempted to standardise the milk collection protocol as far as possible. Although the nurses and I arranged most of our home visits in the morning around 10:00 a.m., some mothers had already collected the milk before we arrived. Most of those mothers did not want to pump their milk in front of others. Studies show that the fat content can be affected by the time of day [274, 276, 277]. As indicated in an observational study, which investigated the breast milk intake and fat content during 24 hours in 71 mothers who were EBF a 1–6-month-old infant, the mean fat content of the milk was significantly higher during the day and the evening (42.8±9.1 and 43.2±9.1 g/L, respectively) compared with the morning and the night (37.1±10.1 and 37.2±10.3 g/L, respectively) (p=0.008). Moreover, although most studies did not find a significant difference in fat production between left and right breast, Mitoulas et al indicated that the amount of fat delivered to the infant was greater from the breast that produced more milk (p<0·005). There is also potential risk that some mothers might satisfy their infants needs first and collect the hindmilk when their infants are full. However, considering a detailed sample collection instruction was provided to them in advance and the importance of collecting the foremilk had been explained by the nurses clearly, this risk was minimised. Moreover, although these methodological issues may lead to variation in fat composition, they should apply equally to both IG and CG and would not necessarily affect the comparison between groups.

The other reason for increased weight gain in IG infants could be increased milk intake. As shown in the MOM study, the mean milk intake measured using stable isotopes of IG infants at 8-weeks was 227 g/d higher than that of the CG infants (P = 0.031) after controlling for gender and milk intake at baseline. However, no significant difference in estimated milk intake between groups was observed in the present study. This might be due to the limited data available, where only 27 mothers provided data suitable for analysis, reducing the power to detect an intervention effect. Originally 36 mothers returned the 48-hour test-weighing form. However, nine mothers did not provide whole-day data on infant weights. As suggested by Kent et al., the breast milk intake of the infant at each feeding was influenced by the time of day, and whether the infant breastfed during the night or not; night breastfeeding can make an important contribution to the total milk intake [276]. Moreover, the milk production has been found to be different between right and left breasts. Mitoulas et al [275] observed that the right breast was more productive (443 vs 356 g/24 hours) and Cox et al [278] also found that the right breast was often more productive than the left. These findings reflect that the milk intake of each single feeding can vary during the day, hence the 24-hour milk intake cannot be reliably estimated if only individual feedings were recorded. In this context, it would be inappropriate to include data from mothers who did not provide records after each feeding during a day. Of the 27 included mothers, three were mixed feeding (IG=2, CG=1). Considering the aim of this analysis was to compare the infant's milk intake between groups, these mothers were included in the analysis and the volume of formula was added when calculating the total milk intake.

Moreover, in the MOM study, a significant increase in milk intake was observed at 12 weeks, which might suggest that a longer duration of listening to the relaxation tape might show more effects on outcomes. In addition, in the MOM study, the milk intake was measured using the more accurate isotope dilution method, whilst in the present study, only test-weighing was applied due to the unsuccessful ethical application when proposing the use of isotopes. Test-weighing data were adjusted using a 5% insensible water loss (IWL) based on the published literature [209], instead of using an exact value measured by extra measurements for each infant; given this, the variance of water loss between infants could not be identified [279]. Further studies are needed to confirm the effects of relaxation therapy on infant milk intake, preferably with more accurate measurements, such as the established isotope dilution measurement. Moreover, it should be noted that in further studies with a larger sample size, formula intake could be a potential confounder if included in the total milk intake and, subgroup analysis is suggested when comparing the total milk intake between groups.

Apart from the potential effects of relaxation therapy on changes of breast milk fat content and energy, other potential factors such as effects on breast milk hormones, and microbial community structures may also be mediators involved in mother-infant signalling. Whereas milk hormone assessment was not conducted in the present study, the microbial community structures in breast milk, maternal and infant gut were explored in the study and are discussed in the next chapter.

6.6.5. Infant behaviours

Of the 51 mothers included in the analysis, 11 did not follow the instruction and recorded two or more behaviours on the same time scale. For example, “feeding” “awake/happy” “playing” appeared at the same time, and “colic” appeared together with “crying” or “fussy”. Although this could be plausible, the overlapping recordings resulted in errors in the final calculation; when adding the duration of each recorded behaviour the sum was greater than 1440 minutes per day. Hence, to reduce the reporting error, infant time spent on crying, colic, and fussing were merged as “distress”, and the “feeding” “awake/happy” and “playing” time were merged as “awake (happy)”.

Inconsistent with the MOM study, which found significantly longer sleeping in IG infant at 6-weeks, no significant difference was found in any of the assessed infant behaviours. Pearson correlation results showed that lower maternal stress at 8-weeks was significantly correlated with longer awake (happy) duration per day and more frequent awake & happy behaviours. However, while the IG mothers had significantly reduced stress compared to the CG, no significant differences were found between groups regarding the awake (happy) duration and frequency. As hypothesised in chapter 2, by reducing the tension between mother and infant, the infant may benefit more from the increased investment from mothers by consuming more milk or milk with optimised composition. This could further result in more favourable infant behaviour, such as longer sleeping time and reduced crying behaviour, since the infant could obtain energy more efficiently from breastfeeding and does not need to frequently demand food. One

reason for the non-significant result in my study might be the limited number of returned dairies and the relatively low quality of several dairies. When looking at the mean value of infant sleeping and distress in IG and CG, the IG infants showed higher mean values for sleeping and lower mean of “distress”, so it is plausible that the small number of mothers reduced the power to detect effects.

A number of studies have suggested that maternal psychological status could affect infant behaviour; on the one hand, postpartum depression and anxiety may increase infant salivary cortisol and result in negative infant emotionality [280-283]. A longitudinal study investigated mother–infant interaction from birth to 9-months postpartum in 971 women with reported anxiety and depression symptoms after childbirth. Results showed that infants of depressed mothers presented poorer social engagement, lower mature regulatory behaviours, more negative emotionality, and highest cortisol reactivity [281]. Further, experimental studies found an acute effect of a relaxation intervention on reducing cortisol level in maternal breast milk. Varişoğlu and Güngör reported the cortisol levels in mothers who received music therapy were significantly lower post intervention compared to pre-intervention [120]. In the MOM study, the cortisol concentration was reduced 34% from fore to hind milk and the reduction was significantly greater in the IG than that in CG mothers [124]. However, the reduction was not significant during subsequent home visits, which, as suggested by the author, may be due to the reduced sample available for the analysis. Hence, further research is needed to examine the long-term effects of using relaxation techniques on

reducing breast milk cortisol and whether the reduced milk cortisol is a mediator between a relaxed mother and more positive infant behaviour.

On the other hand, evidence showed that relaxed mothers are more receptive to their babies' facial expression compared to mothers who are stressed; mothers with postnatal anxiety or depression have difficulties in understanding the infant's behavioural cues and find it difficult to respond appropriately to their infants [284-286]. A systematic review including 14 studies further suggested that mothers with depression and anxiety after birth were more likely to identify negative emotions in infant faces, and were less accurate when identifying positive emotions, such as happiness, in their infant's face [287]. A clinical trial showed that mothers with depression presented blunted responses to their own infant's distress faces in the dorsal anterior cingulate cortex; moreover, mothers with higher levels of current symptomatology presented reduced responses to their own infant's joy faces in the orbitofrontal cortex and insula [288]. Given these findings, mothers who were more relaxed may have been more sensitive to their infant's cues, which could have been reflected in their diary entries and resulted in the significant association between maternal stress and infant awake (happy) duration and frequency.

Based on the discussion above, subjectivity can be one of the limitations of the 3-day diary. As a result of reduced stress, mothers in the IG may be more likely to capture positive behaviours in their infants. Hence, objective measurements for infant behaviour assessment have been suggested, for example, a computerised audio-visual

event recording system was applied in premature infants for monitoring infant position and the wide variety of behaviours associated with bradycardic events [289]. Studies also used audio recording for the assessment of crying, fussy, and colic behaviours [290, 291]. However, such monitoring instruments might not be accepted by many mothers. Another way to obtain more accurate infant behaviour records is to develop an electronic behaviour recorder that can be easily used on phones. Since a number of mothers in my study complained that there was too much paperwork for them to complete, this might be a good option. Electronic diaries have been designed to capture the 24-hour infant and caregiver behavioural data in previous studies. Lam et al. used Palm Tungsten E (Palm Inc., Milpitas, CA) for the measurement of infant behaviour along with a paper dairy [292]. This product has a coloured screen and a 64 MB memory card for backup purposes. The main screen displayed entered behavioural data in the diary component. Participants could move between each by clicking on 1 of 3 boxes on the top right corner of the screen. While this method could be valuable to adapt for future studies, an App with similar design might be even more convenient for mothers to use on their phone. It could also avoid the overlap of time for several behaviours if it was designed to only allow one behaviour to be recorded at a time. An App could also be made attractive to encourage compliance. However, although this approach could improve compliance, it would not really get around the problem of maternal perception and subjectivity in reporting their infant's behaviour.

6.6.6. Strengths and limitations of this study

The present study is the first randomised controlled trial investigating the effects of relaxation therapy on primiparous mothers and their infants born late preterm and early term. Moreover, it is the first study in the Chinese population with a distinctive cultural background. Compared to previous intervention studies in preterm infants (summarised in Chapter 2, Table 1), this study assessed the outcomes from both psychological and physiological aspects in mother and infant dyads, instead of focussing on one aspect. Compared to the MOM study, the present study used a larger sample size, and recruited mothers who may be more stressed following late preterm/early term delivery, which I hypothesised might lead to a greater intervention effect. In addition, the study benefitted from stringent pre-study training, frequent validation of instruments and procedures, together with randomisation assignment, and although these could not guarantee objectivity, they are approaches by which errors were minimised and internal validity was strengthened. Another strength was that mothers were blinded to the use of relaxation therapy, being told only that the aim of this study was to investigate maternal breastfeeding outcomes following late preterm/early term delivery. This may have reduced the risk that mothers in the control group would seek alternative interventions to relax. Last but not least, the present study had good follow-up rates for all primary outcomes and a good compliance with the intervention, so dose-response effects could be assessed based on the intervention usage dairy.

As shown in Table 6.14, some limitations in this RCT that should also be acknowledged. First, due to the nature of the study, IG mothers needed to scan the QR code during the home visit, so the research assistant and I could not be blinded to the intervention group. One solution to this issue would have been for an independent research assistant to conduct the randomization allocation, provide the relaxation tape and diary prior to the first home visit, and collect it prior to the 8-week home visit. However, the additional interruption to mothers during this vulnerable period was considered unethical and impractical.

Second, the present study involved several research assistants for data collection. Although I tried to go with them during most of the home visits, and regular training was arranged every month, it is impossible to be sure that the standard operating procedures for infant measurements and milk sample collection were followed consistently.

Moreover, although the protocol stated that infant anthropometry measurements would following WHO guideline including that the infant should be weighed naked, in reality, most mothers refused to completely remove their infant's clothes. Therefore, the actual measurement included light clothes (such as a vest) or a small cotton cloth for those who removed clothes, and a dry nappy. This may have resulted in an overestimation of the weight SDS. However, the measurement was performed in a consistent manner at 1-and 8-week home visits, so the changes of SDS (the primary outcome) should be less influenced. Moreover, the clinical nurses who conducted the

measurements had no investment in the outcome of the study so it is probable that any bias was consistent in the IG and CG.

Further, considering the relaxation intervention is a meditation audio, there is a possibility that the infant also received the relaxation mediation if the audio was broadcast around the room. This could be a confounding factor when interpretating the mother-infant interactions. Unlike music therapy, the most effective part of the meditation may be the content of the audio, which could not be understood by the infant, although the soft voice of the speaker may also have relaxation effects. Moreover, some mothers may have preferred to use headphones when listening to the music on their phone; in this case the infants would not have been exposed to the intervention. Nevertheless, I did not ask the mothers to record how they listened to the audio. Future studies should ideally avoid the exposure of infants to the intervention when investigating mother-infant signalling. Other limitations were indicated in previous sections along with the discussion of results. **Table 6.14** provides a brief view of the main limitation for each outcome.

Table 6.14. Summary of outcomes and corresponding limitations.

Variables	Between group differences			Limitations
	1-week	8-weeks	Changes	
1 Maternal psychological changes [a]				
Stress	N	S	S	Response bias (self-reported)
Anxiety	N	N	N	Response bias (self-reported)
2 Infant Growth				
Weight gain	N	S	S	Not measured at the same time of the day; The typical home visit was to include a vest or light cotton cover and dry nappy during weight measurement.
Length gain	N	N	T [b]	Potential risk for inter-operator error
3 Macronutrient composition in breast milk				
Fat	N	N	T	1. Not measured at the same time of the day; 2. Maternal diet not controlled for or recorded.
Protein	N	N	N	
Carbohydrate	N	N	N	
4 Milk energy	N	N	T	Not measured at the same time of the day.
5 Milk intake	N	N	N	1. Low response rate; 2. Potential risk for inter-operator error; 3. ISW using the published data instead of accurate measurement.
6 Maternal feeding attitude and infant eating behaviour [a]				
IIFAS	N	N	N	Response bias (self-reported)
BEBQ	N	N	N	Response bias (self-reported)
7 Infant behaviours				
Sleeping	-	N	-	1. Low response rate and quality; 2. Response bias (self-reported) 3. Recall bias
Awake (happy)	-	N	-	
Distress	-	N	-	

Notes: N=not significant; S=significant; T=non-significant trend; ISW=insensible water loss

[a] These outcomes were assessed using self-reported questionnaire, response bias refers to mothers who listened to the relaxation therapy might expect themselves to be affected after the intervention.
[b] The non-significant trend in length gain was only observed when using the 21st intergrowth SDS.

6.7. Conclusion

Overall, the present study showed significant effects of the relaxation intervention on reducing maternal stress and increasing infant weight gain after birth. Mothers of girls showed greater exposure to the intervention compared to mothers of boys, which might be related to the finding that the weight gain in the IG was greater in girls than in boys. Non-significant trends of higher breast milk fat and energy were observed in IG mothers, which were also greater in mothers of girls than mothers of boys. Mothers who were less stressed and more relaxed may invest more in their infants by producing milk with more favourable nutritional composition and higher energy content. In this context, findings of the study supported my hypotheses. Given the differences in milk composition and increased energy content, the infant was hypothesised to have reduced crying and longer sleeping durations. However, no significant differences were found between groups regarding infant behaviours; this possibly reflects the smaller sample of mothers who provided data on this outcome, which limited the power to detect effects. Further investigations are suggested to confirm the potential beneficial effects of the intervention. In the next chapter, I will present results of the effects of relaxation therapy on microbial community structures in maternal and infants' gut, as well as in breast milk. I will also discuss the possible role of the microbiota in mother-infant signalling.

Chapter 7

Effects of Relaxation Intervention on Microbiota Composition in Maternal Breast Milk, Maternal and Infant Gut

The microbiome analyses of my research investigated whether the microbiome is one of the factors in mother-infant signalling which can be impacted by the relaxation intervention. The microbiome composition and diversity in maternal breast milk, gut, and infant's gut were examined and compared between intervention and control groups to explore the chain of signals from mother to infant. Maternal breast milk (BM), maternal stool (MS), and infant stool samples (IS) were collected at 1-week and 8-week home visits. The 16S rRNA amplicon sequencing technology was used to identify the microbial community structures in breast milk and maternal and infant's gut (by examining their stool samples).

This chapter starts with a brief introduction about amplicon-based sequencing of the 16S rRNA gene and the concept of operational taxonomic units (OTU), followed by the research methodology for the microbiome analyses, and the results obtained using 16S rRNA amplicon sequencing technology. A total of 92 mothers (IG=47, CG=45) provided at least one of the three samples at the 1-week home visit, whilst 86 mothers provided at least one of the three samples at 8-weeks. Only participants who provided all three

samples at both time-points were included. In addition, to control for the known effect of delivery mode on the infant microbiota, participants were only included if they delivered vaginally. Given this, a total of 38 mother-infant pairs (IG=19, CG=19) with 228 samples (six samples per mother-infant pair) were included in the analysis presented in this chapter. The 228 samples were transported to Novogene Technology Inc. in Beijing, China, where DNA extraction and sequencing of the 16S rDRNA were conducted. The remaining samples were stored at -80^{o}C at the BCH Lab for future investigation.

Contribution statement:

The DNA extraction and 16s rDNA amplicon sequencing was conducted by Novogene Technology Inc. in Beijing, China (Novogene). For the statistical analysis, I conducted the independent t-test/Wilcoxon rank-sum test for group comparison of alpha-diversity; the remaining bioinformatics analyses (e.g. Anosim, MetaStat) were conducted by Novogene.

Due to the Covid-19 pandemic, the Chinese government implemented a new policy that all international collaboration research involving the analysis of human genetic information should apply for national approval from the Ministry of Science and Technology of China before the data could be shared. The complete results of the microbiome analysis were not obtained as the application for national approval is still pending. Hence, only preliminary results with a fixed format for some figures are

presented in this Chapter. The whole results of the planned analysis will be reported in separate paper for publication after obtaining the national approval.

7.1. Introduction

7.1.1. Overview of the Bacterial 16S RNA

In recent years, amplicon-based sequencing of the 16S rRNA gene has enabled the analysis of hundreds of samples from different origins at high phylogenetic resolution and much greater depth (to genus level) than previously possible. 16S rRNA is located on the small ribosomal subunit of prokaryotic cells, including 10 conserved regions and 9 hypervariable regions. Among them, the conserved regions have little difference among bacteria, and the hypervariable regions have genus or species specificity, which vary with different genetic relationships. Therefore, 16S rDNA can be used as a characteristic nucleic acid sequence to identify biological species and is considered to be the most suitable index for bacterial phylogeny and taxonomic identification. 16S rDNA amplicon sequencing usually selects one or several variant regions, designs universal primers with conserved regions for PCR amplification, and then carries out sequencing analysis and strain identification for hypervariable regions. To investigate the species composition of each sample, the effective tags of all samples are clustered by OTUs (operational taxonomic units) with 97% identity, and then the sequence of OTUs is annotated.

7.1.2. Concept of the OTU

After sequencing, a huge number of bacteria at several classification levels (phylum, class, order, family, genus) are observed for each sample. Although species annotation

can be performed for each sequence, the comparison process is time-consuming, and errors in the process of amplification and sequencing will reduce the accuracy of the comparison results. Therefore, the concept of OTUs (operational taxonomic units) was introduced [293]. Firstly, the sequences are clustered according to a certain degree of similarity; each class formed is called an OTU. Species annotation is carried out based on taxon (OTU); that is, a representative sequence from the OTU is selected and compared with the database to obtain the classification status information of the OTU. The use of OTUs not only simplifies the workload and improves the analysis efficiency but also can remove some wrong sequences, such as chimera sequences, and improve the accuracy of analysis.

7.1.3. Research aims and hypotheses

The aim of the analyses presented in this chapter were:

1. To test the hypotheses that the use of relaxation therapy by breastfeeding mothers following late preterm and early term delivery from 1-to 8-weeks postpartum will result in altered microbiome diversity in maternal gut and breastmilk, as well as in the infant's gut in IG mothers;
2. To compare the phylum level microbiome composition changes from 1-week to 8-weeks in IG and CG.

7.2. Materials and Methods

Breast milk and stool samples from mothers were collected during home visit following standard proposal as stated in Chapter 4. Stool from infants' soiled diapers were collected by nurse or by mothers during home visits. All samples were collected into sterile tubes and stored at -80^{o}C at the BCH Lab. Samples for inclusion of the microbiome analysis were transported to the laboratory of Novogene Technology Inc. (Beijing, China) where the DNA extraction and 16S rRNA sequencing were performed. Independent research assistants at Novogene who were blinded to the randomisation status of the subjects conducted the analysis. Detailed methods and materials they used are presented in Appendix 5.

7.2.1. Alpha diversity and Beta diversity

In order to compare the bacterial diversity in samples, a variety of bioinformatics tools have been developed. Alpha diversity is used to analyse the microbial community diversity within the sample. Shannon and Simpson diversity indices are commonly applied in bacterial diversity measurement based on OTUs [294], whereas Chao1 indices and Abundance-based Coverage Estimator (ACE) are used to estimate richness (estimated richness; measurement of OTUs expected in samples given all the bacterial species that were identified in the samples) [295]. Currently, there is no general agreement on which diversity index is the best to use [296], hence, all four indicators were tested in the current project.

While alpha diversity is estimated for each participant separately, beta diversity is an inter-individual measure that examines similarity of communities relative to the other samples analysed (**Figure 7.1**). The dimension reduction technique, Non-Metric Multi-Dimensional Scaling (NMDS), was employed to visualise data on a smaller number of axes, whereby samples closer together are more similar in their microbial composition. Statistical differences were measured by using Bray Curtis distance.

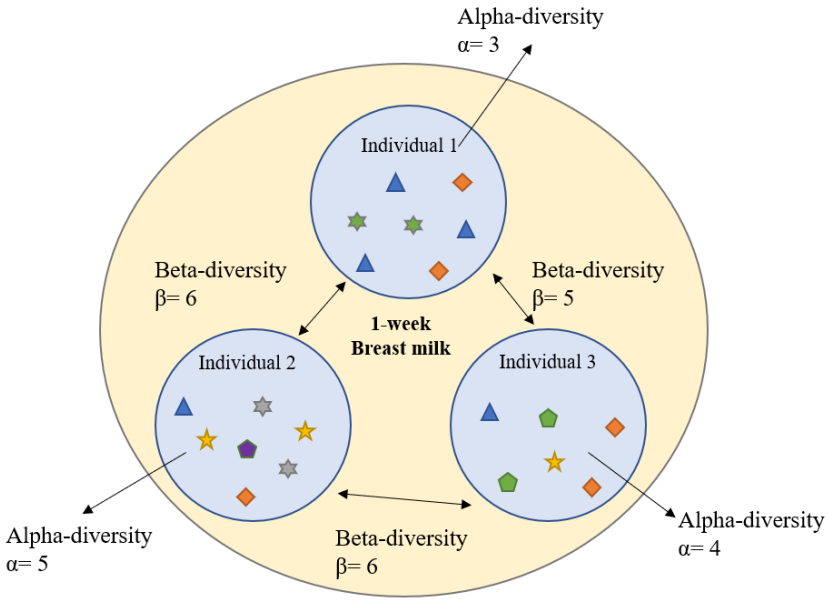


Figure 7.1. Conceptual representation of alpha and beta diversity.

Notes: Alpha diversity describes the species diversity or species richness of a sample (for example, the 1-week breast milk sample) within an individual. Beta diversity describes the species diversity of a sample between individuals.

7.2.2. Statistical Analysis

Statistical analyses were conducted using R software (version 4.1.2) by Novogene and checked in SPSS (version 26.0) by myself (JY). The BM, MS and IS samples in IG and CG at 1-and 8-weeks were all analysed (total n=228). Rarefaction curve and species accumulation boxplots were performed to check if the sample size was sufficient. Alpha and beta diversity index were calculated using QIIME (version 1.9.1). The differences in alpha diversity index between IG and CG or between 1-week and 8-weeks samples were examined by t-test (parametric data) or Wilcoxon rank-sum test (non-parametric data) (SPSS version 26.0) using observed species, Shannon, Simpson, Chao1, and ACE indices. Differences in beta-diversity are presented in Nonmetric Multidimensional Scaling (NMDS) based on Bray–Curtis distance; the NMDS was used to visualize the differences in microbial composition. Statistical difference of beta-diversity between groups was test using analysis of similarities (Anosim) based on Bray-Curtis distance. Further, MetaStat was used to detect the differences in bacteria at different classification levels between groups.

It should be mentioned that statistical analyses were carried out by Novogene at each classification level (phylum, class, order, family, genus). However, at the time of completion of this thesis, the whole data were not released by Novogene[*], and only part of the results at phylum level are presented in the Results Section. **Table 7.1** outlines

[*] This was due to a Covid-related issue which is described in the separate Covid impact statement accompanying this thesis

the coding methods of the tested sample groups and **Table 7.2** outlines the statistical analyses and the purpose of each test used.

Table 7.1. Coding of the tested groups

Tested group samples		Control group	Relaxation Group
Breast milk samples	1-week	C.BM1	R.BM1
	8-weeks	C.BM8	R.BM8
Maternal stool samples	1-week	C.MS1	R.MS1
	8-weeks	C.MS8	R.MS8
Infant stool samples	1-week	C.IS1	R.IS1
	8-weeks	C.IS8	R.IS8

Notes: C=control group; R=relaxation group; BM=breast milk sample, MS=Mother's stool sample, IS=infant stool sample; 1=1-week; 8=8-weeks.

Table 7.2. Statistical tests used to analyse the microbiota data.

	Aims of the analysis	Function
Independent t t-test	Detect different species between independent groups at each classification level (phylum, class, order, family, gene, disciplines).	Detecting species differences between groups
Wilcoxon rank-sum test	Detect different species between independent groups at each classification level, for variables that were not normally distributed.	Detecting species differences between groups
Anosim [a]	Similarity analysis, which is mainly used to analyse the similarity between high-dimensional data groups and provide a basis for the evaluation of the significance of differences between data	Detecting microbial community structure difference between groups
MetaStat [b]	Using hypothesis test on the species abundance data between groups to obtain the p value; then, the Q value[a] was obtained by correcting the p value; based on the Q-value, the species with significant differences are identified	Detect the abundance differences in microbiome between two groups.

Notes: [a] The results of Anosim provide an R-value. R-value is between (- 1, 1). R > 0 indicating significant difference between groups; R < 0 indicating that the difference within the group is greater than the difference between groups; R=0 indicating that there is no significant difference between groups and within groups. The reliability of statistical analysis is expressed by p-value, and P < 0.05 indicates statistical significance. [b] Q value is a correction of p-value using Fisher test; it is the measurement of false discovery rate, with significance Q<0.05.

7.3. Results of the species abundance at phylum level

7.3.1. Overview of the sample

A total of 228 samples from 38 mother-infant dyads at 1- and 8-weeks were collected for 16S rRNA sequences, in which 21,629,194 qualified sequences (effective tags) from 30,947,688 raw sequences (raw tags) were filtered. The mean maternal age was 31±3.8 in IG and 30±1.6 in CG. Eight of the 19 mothers in IG and 13 of the 19 mothers in CG were EBF, with no significant difference between groups. Other baseline characteristics were not significantly different between IG and CG (Appendix 6). As shown in the species accumulation boxplot (**Figure 7.2**), the curve flattens when the number of samples increased to over 222, which indicates that the amount of sequencing data from the 228 samples available in the present study was sufficient, and that more data would only produce a small number of new OTUs. Similar results were shown in the rarefaction curve and can be found in Appendix 7.

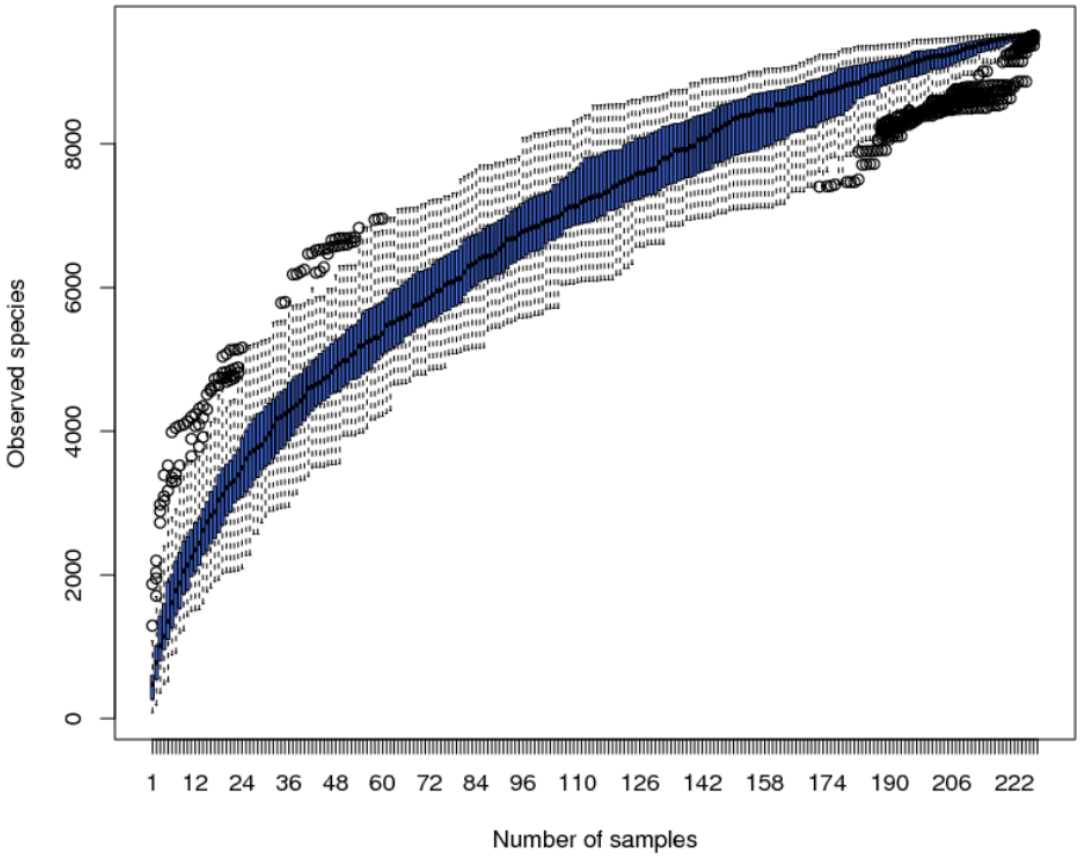


Figure 7.2. Species accumulation boxplot.

Notes: X-axis refers to the sample size; Y-axis is the number of OTUs after sampling. When number of samples was small (left of the X-axis), the sharp rise of the position of the box chart indicates that the sample size is insufficient and needs to be increased. When the number of samples increased (right of the Y-axis), the position of the box chart tends to be flat, which means that the species in this environment will not increase significantly with an increase in sample size. In other words, it means the sampling is sufficient and data analysis can be carried out.

7.3.2. Differences in microbiome diversity between IG and CG

7.3.2.1. Alpha diversity differences between IG and CG in BM, MS, and IS samples

Alpha diversity differences between IG and CG samples were analysed using Wilcoxon rank-sum test based on median value. Observed species and other indices (Shannon, Simpson, Chao 1, and ACE) were compared between IG and CG among BM, MS, and IS samples at 1-week and 8-weeks. Results of the analysis are presented in **Figure 7.3** and **Table 7.3.** At baseline (1-week), there was no significant difference in any of the sample types between IG and CG for any of the assessed indices; at 8-weeks, the median value of observed species, Chao1 and ACE was significantly lower in BM and MS samples and significantly higher in IS samples in the IG, compared to CG.

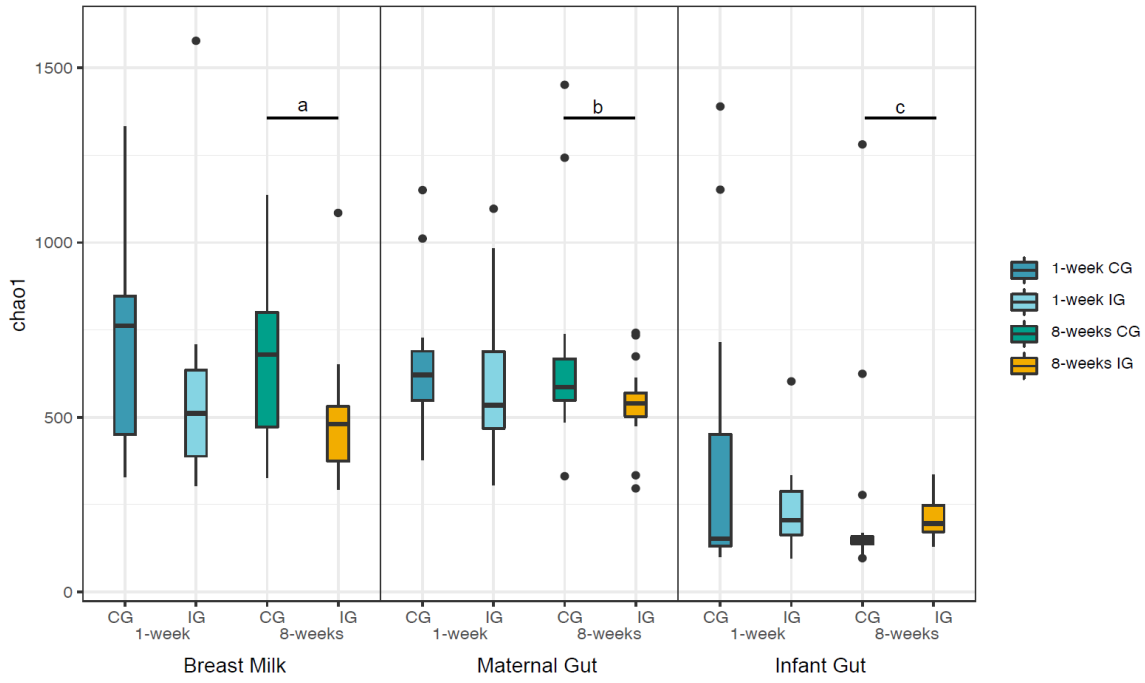


Figure 7.3. Alpha diversity differences between IG and CG in BM, MS, and IS samples.
Notes: IG=intervention group; CG=control group; BM=breast milk; MS=maternal stool; IS=infant stool. The box chart presents the median, dispersion, maximum, minimum and outlier values of species diversity in each group. [a] p=0.012; [b] p=0.040; [c] p=0.004. Maternal and infant gut microbiome were examined using their stool sample. Similar trends and results were obtained when using observed species, Chao1, and ACE indices; this chart is based on results of Chao1 indices. Outliers are shown as spots in the figure.

Table 7.3. Alpha diversity in breast milk, maternal gut, and infant gut microbiota and comparison between IG and CG.

				O_S	Shannon	Simpson	Chao1	ACE
1-week	Breast milk	Median	CG	669	3.52	0.80	762	780
			IG	448	4.07	0.86	511	521
		Wilcoxon		128	181	203	131	128
		Sig. (2-sided)		0.13	1.00	0.53	0.15	0.13
	Maternal gut	Median	CG	570	5.84	0.96	621	614
			IG	501	5.59	0.94	534	543
		Wilcoxon		142	154	145	139	137
		Sig. (2-sided)		0.27	0.45	0.31	0.23	0.21
	Infant gut	Median	CG	141	2.79	0.75	153	152
			CG	185	2.95	0.74	205	213
		Wilcoxon		204	204	197	200	202
		Sig. (2-sided)		0.51	0.51	0.64	0.58	0.54
8-weeks	Breast milk	Median	CG	591	4.27	0.87	679	698
			IG	438	3.86	0.84	480	481
		Wilcoxon		101	130	155	94	90
		Sig. (2-sided)		**0.018**	0.15	0.47	**0.011**	**0.008**
	Maternal gut	Median	CG	554	5.81	0.96	586	591
			IG	508	5.68	0.95	540	548
		Wilcoxon		109	140	141	110	98
		Sig. (2-sided)		**0.034**	0.23	0.25	**0.040**	**0.015**
	Infant gut	Median	CG	134	2.78	0.74	147	149
			IG	173	3.08	0.77	196	198
		Wilcoxon		282	238	230	280	285
		Sig. (2-sided)		**0.002**	0.10	0.15	**0.003**	**0.002**

Notes: IG=intervention group; CG=control group; O_S=number of species visually observed (number of OTUs). Maternal and infant gut microbiome were examined using their stool sample. Significance tested using Wilcoxon Rank-Sum Test

7.3.2.2. Beta diversity differences between IG and CG in BM, MS, and IS samples

Beta diversity of the microbiome was examined using the NMDS plots based on Bray-Curtis distances (**Figure 7.4**); results showed that the microbiome compositions were very diverse among BM, MS, and IS samples. Comparison of the beta-diversity between IG and CG was performed using Anosim. Whilst no significant difference found in any of the three sample types between IG and CG at 1-week, a significant difference was observed in maternal stool samples at 8-weeks ($p=0.001$). However, because the complete data have not yet been released from Novogene, it is not possible to describe in detail the nature of the differences between the IG and CG. No significant differences were found in BM and IS between IG and CG at 8-weeks.

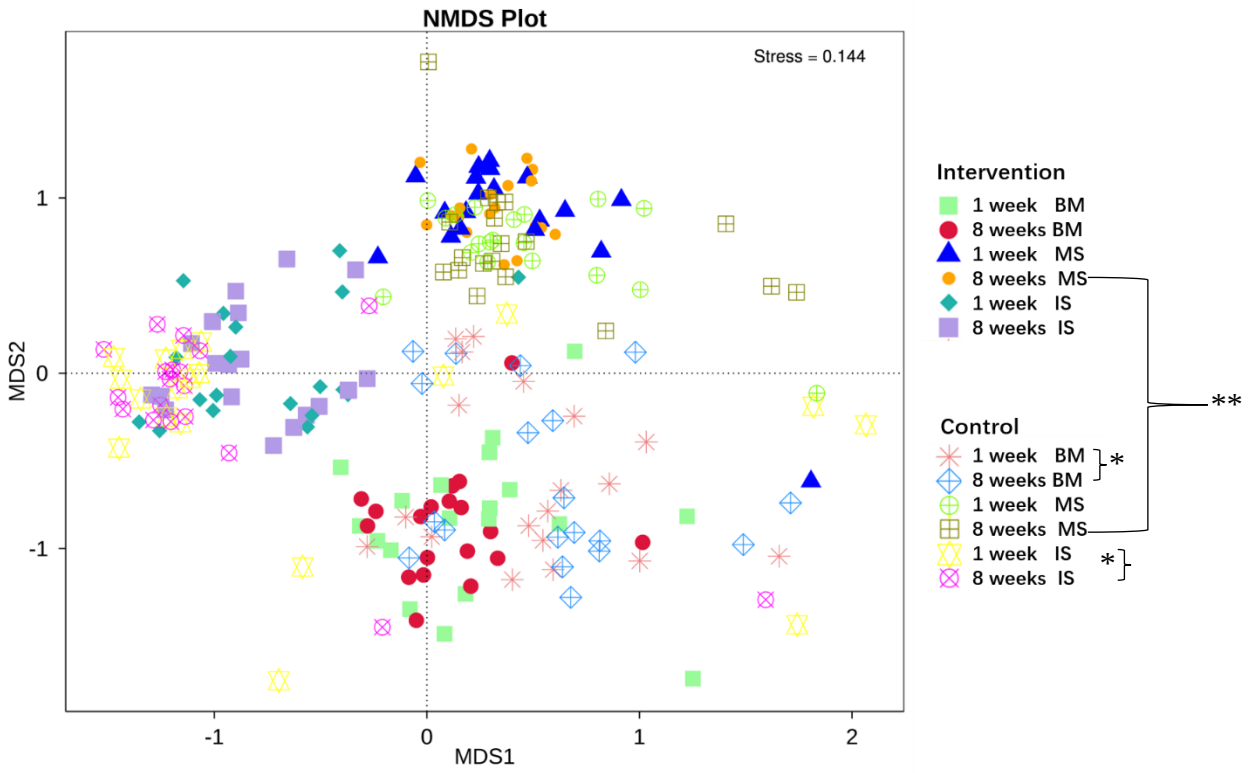


Figure 7.4. Beta diversity differences between IG and CG in BM, MS, and IS samples using Bray-Curtis distances.

Notes: NMDS=Non-Metric Multi-Dimensional Scaling; BM=breast milk; MS=maternal stool; IS=infant stool. Each point in the figure represents a sample, the distance between points represents the degree of difference. When the value of "stress" is less than 0.2, it means the NMDS can accurately reflect the degree of difference between samples. *p=0.039, **p=0.012, *** p=0.001, tested by using Anosim. This Figure was provided in the brief report from Novogene. Currently, the raw data of Bray-Curtis distance matrix has not been released, so it is not possible to present more detailed information on the beta-diversity.

7.3.3. Microbiome diversity changes from 1-week to 8-weeks

As shown in **Figure 7.5**, no significant changes of alpha-diversity from 1-to 8-weeks were found for BM, MS, and IS samples. The beta-diversity was significantly different between 1- and 8-weeks for BM and IS samples in the CG, whereas in the IG, the beta-diversity was not significantly different between 1- and 8-week in BM, MS, and IS samples (**Figure 7.4**).

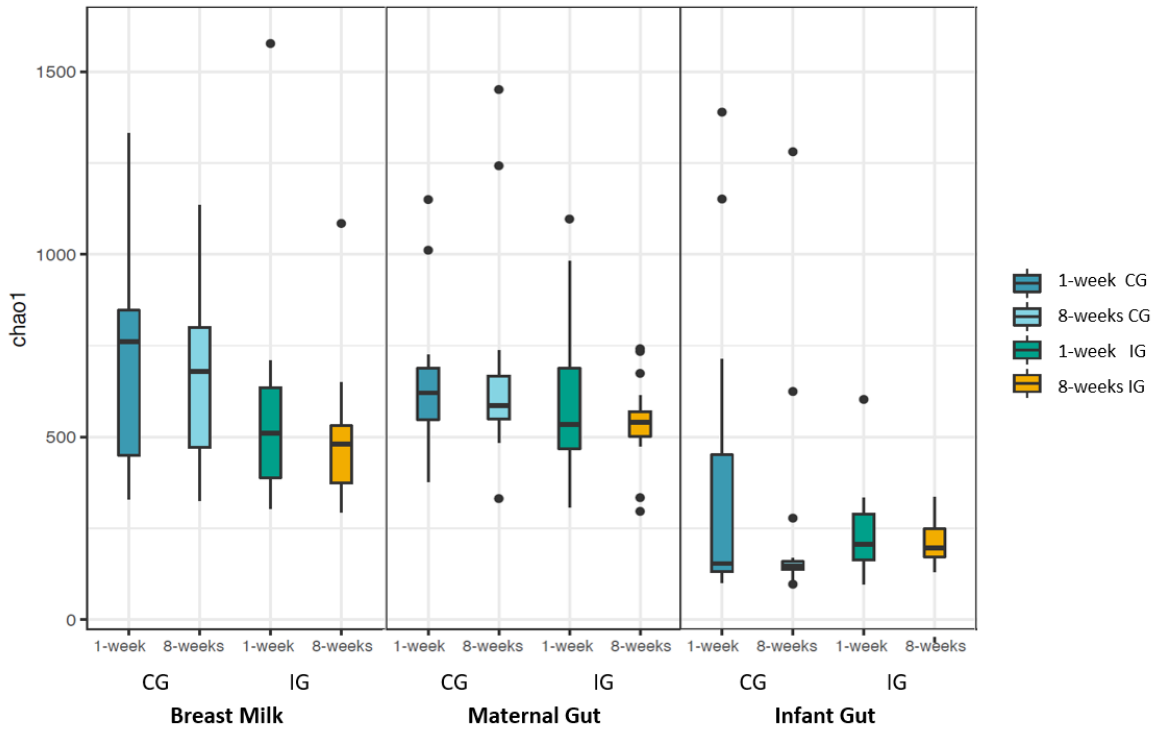


Figure 7.5. Alpha diversity differences between 1- and 8-weeks in BM, MS, and IS samples.

Notes: IG=intervention group; CG=control group. The box chart presents the median, dispersion, maximum, minimum and abnormal value of species diversity in each group. IG=intervention group; CG=control group. Maternal and infant gut microbiome were examined using their stool sample. This figure uses the same data from the alpha diversity analysis as Figure 7.6, but compares 1-week and 8-week samples.

7.3.4. Changes of microbiome composition at phylum level from 1-week to 8-weeks

The top ten OTUs with the largest abundance in BM, MS, and IS samples are shown in Figure 7.6. *Firmicutes* and *Proteobacteria* were the most dominant bacteria in maternal breast milk and infant stool samples at 1- and 8-weeks, whilst *Firmicutes* and *Bacteroidetes* were the first and second most abundant bacteria in maternal stool samples at 1-and 8-weeks. *Actinobacteria* and *Bacteroidota* were the third and fourth most abundant bacteria in infant stool at both 1-and 8-weeks.

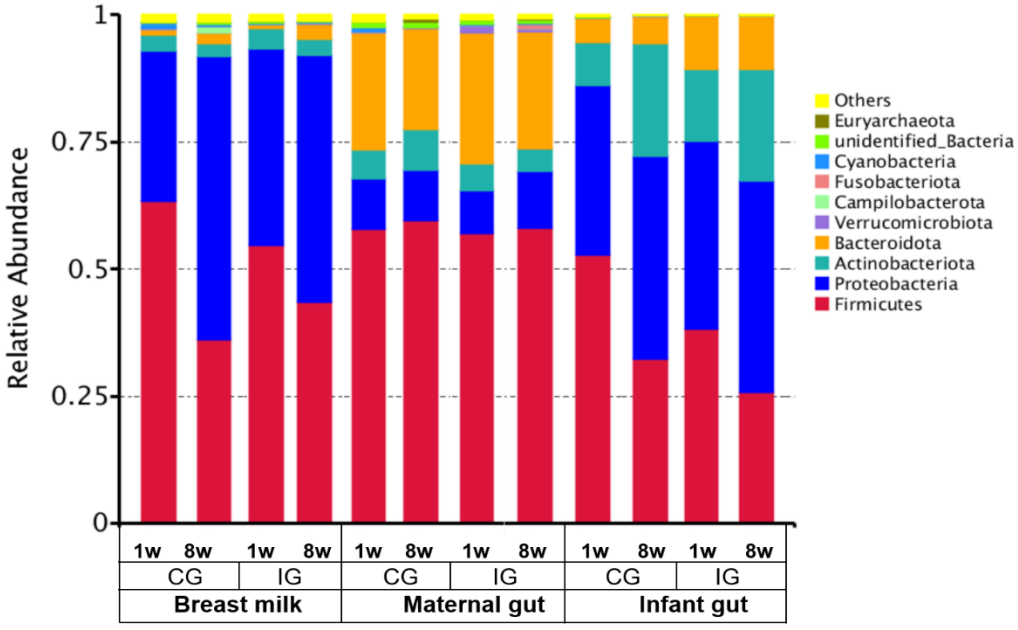


Figure 7.6. Relative abundance of OTUs among group of samples.

Notes: 1w=1-week; 8w=8-weeks; IG=intervention group; CG=control group. Maternal and infant gut microbiome were examined using their stool sample.

As shown in **Figure 7.6**, from 1- to 8-weeks there was an increase in *Proteobacteria* and *Actinobacteria* in breast milk and infant stool samples, whereas *Firmicutes* decreased from 1-to 8-weeks in both sample types. Independent t-test and MetaStat were applied to identify the significance of the changes mentioned above but unfortunately, at the time of completion of this thesis, only *Firmicutes* and *Proteobacteria* results were released. These results showed that from 1- to 8-weeks, the decrease in *Firmicutes* and the increase in *Proteobacteria* in breast milk were statistically significant in the CG, but not in the IG (**Figure 7.7** and **Figure 7.8**). Although not significant, the decrease in *Firmicutes* in CG infant gut was more obvious than for the IG (**Figure 7.6** and **Figure 7.8**). Moreover, although I do not have statistical analysis results for other bacteria as yet, the *Actinobacteria* in infant gut appeared to be higher at 8-weeks than at 1-week in both IG and CG.

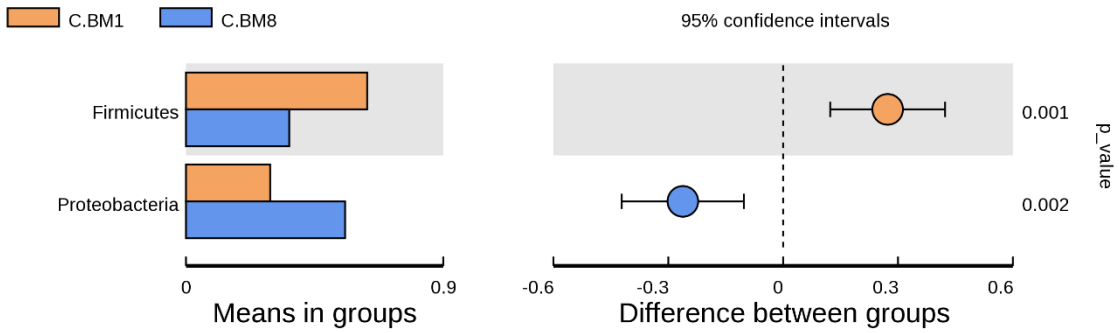


Figure 7.7. Difference in the abundance of Firmicutes and Proteobacteria between 1-week and 8-week breast milk samples in the control group (t-test).
Notes: C=control group, BM=breast milk sample, 1=at 1-week collection, 8=at 8-weeks collection.

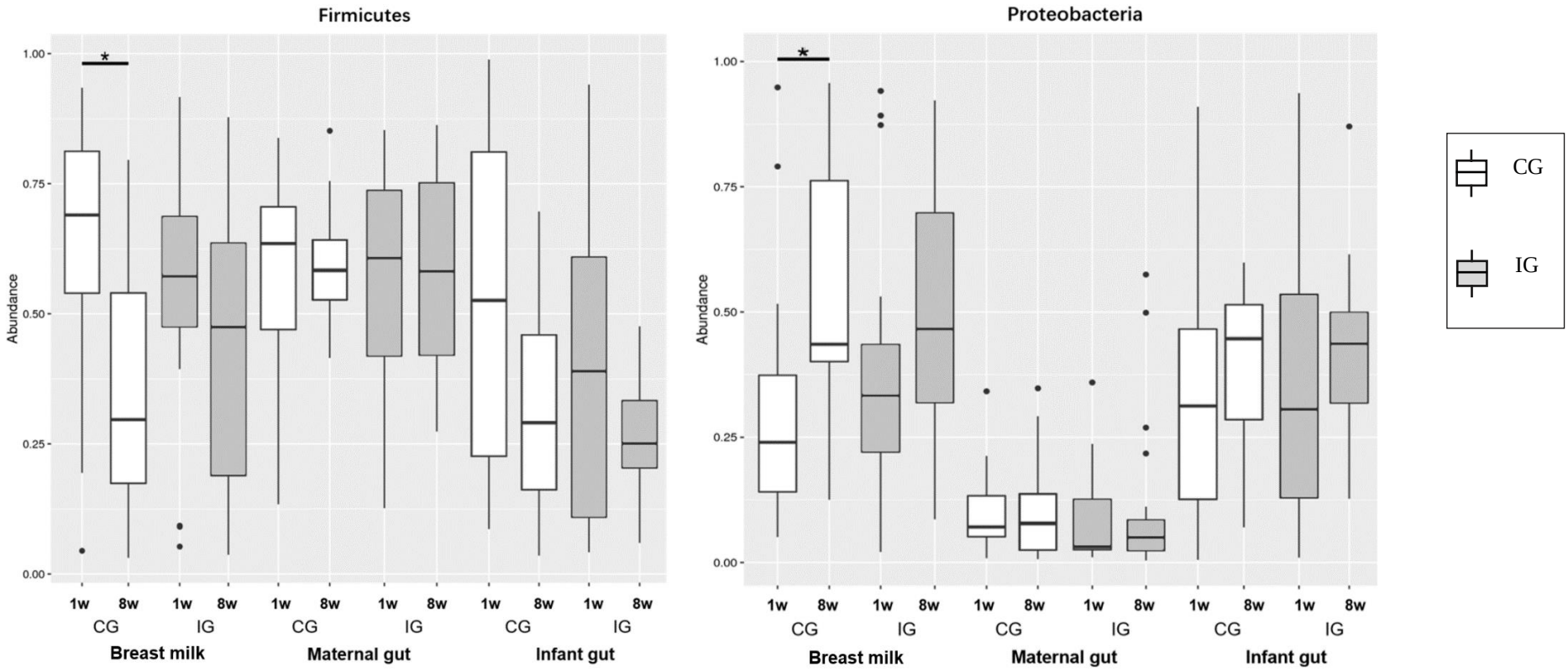


Figure 7.8. Difference in the abundance of Firmicutes and Proteobacteria between IG and CG.

Notes: IG=intervention group, CG=control group. The X- axis is the sample grouping; the Y-axis is the relative abundance of the corresponding species. X-axis represents two groups with significant differences. If there is no line, it indicates that there is no difference between the two groups. "*" indicates significant difference between the two groups (Q value < 0.05). Maternal and infant gut microbiome were examined using their stool sample.

7.4. Discussion

7.4.1. Summary of the results

Table 7.4 summarises the significant results from the microbiome analysis that were available at the time of writing this thesis. While no significant differences were found at baseline between IG and CG, at 8-weeks the alpha diversity was significantly lower in maternal gut and breast milk, and significantly higher in infant gut in the IG than in the CG. The beta diversity in maternal gut differed significantly between IG and CG at 8-weeks. Compared to baseline (1-week), the beta diversity was significantly changed in CG maternal breast milk and infant gut at 8-weeks; whereas no significant changes from baseline to 8-weeks were observed in the IG. Moreover, compared to baseline, there was a decrease in *Firmicutes* and an increase in *Proteobacteria* at 8-weeks in BM and IS samples from both IG and CG, although the change was statistically significant only for BM in the CG. Furthermore, the *Actinobacteria* in infant gut appeared much higher at 8-weeks than at 1-week in both IG and CG, although no statistical comparisons were available from Novogene at the time of completion of the thesis[2]. **Figure 7.9** summarises the current findings in the context of my hypothesis for mother-infant signalling via the microbiome.

1* There was a Covid-related issue in obtaining the full results of the microbiome analyses which is described in the separate Covid impact statement

Table 7.4. Significant results from the microbiome analyses

	IG vs. CG 1-week	IG vs. CG 8-weeks	1-week vs. 8-weeks
Alpha diversity	-	Significantly lower in BM, MS in IG; Significantly higher in IS in IG	-
Beta diversity	-	Significant difference in MS between IG and CG	Significant difference in BM and IS in CG
Firmicutes	-	-	Significantly decreased in BM in CG
Proteobacteria	-	-	Significantly increased in BM in CG

Notes: BM=breast milk; MS=maternal stool; IS=infant stool; IG=intervention group; CG=control group. " – " no significance observed.

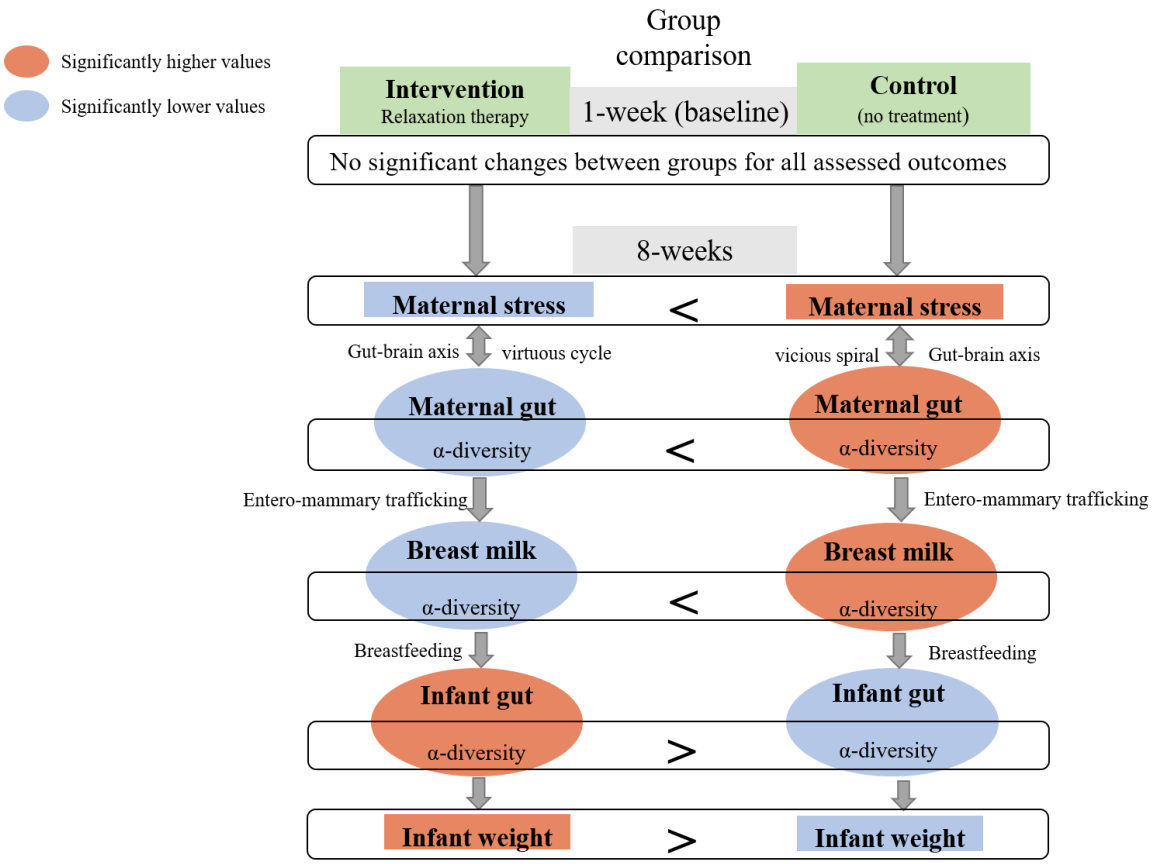


Figure 7.9. Summary of the main findings in IG and CG in the context of my hypothesis on the possible role of the microbiome in mother-infant signalling

Notes: Differences in beta-diversity and composition of Firmicutes and Proteobacteria are not presented in this Figure. Maternal and infant gut microbiome were examined using their stool sample.

7.4.2. Interpretation of the findings from my study

7.4.2.1. Effects of relaxation intervention on microbiome diversity in the mother

Results of my analysis showed that the use of relaxation therapy from 1-to 8-weeks may result in lower alpha diversity in maternal gut and breast milk at 8-weeks compared to CG, while no significant difference was found between groups at 1-week. It has been suggested that a highly diverse microbiome which contributes to functional redundancy may allow individuals to better respond to environmental fluctuations; higher microbiome diversity can also promote intestinal homeostasis and maintain health [297]. However, studies have also reported that higher alpha diversity can be associated with major depressive disorders (MDD) in adults [298]. Similarly, a Chinese case-control study reported that the gut microbiome diversity was significantly higher in postpartum depressive disorder patients than in healthy controls [299]. Carlson et al suggests that increased diversity is not necessarily beneficial for neurocognitive or neuropsychiatric outcomes[300]; one possible explanation is that higher diversity may indicate that fewer resources have been assigned to promote specific gut microbiota with a beneficial impact on neurodevelopment. According to this proposal, the decreased alpha diversity in maternal gut in my study might suggest these women had benefited from the relaxation therapy since the reduced stress may contribute to the lower alpha diversity of their gut microbiome. As mentioned in Chapter 2, there is a bidirectional communication between the central nervous system (CNS) and the GI tract [132, 140], hence the decreased alpha diversity in mothers who received the relaxation therapy

may be a result of their reduced stress as shown in the primary results of my study, but the altered microbiome may contribute to further reduction of maternal stress.

Nevertheless, current findings on the association between the gut microbiota and depression are not consistent. Some studies showed no significant differences in microbiome alpha diversity between MDD patients and healthy controls [301-303], while Huang et al.[304] reported that MDD patients showed significantly lower alpha diversity compared to healthy individuals. As suggested by a systematic review, the relationship between alpha diversity and psychological problems can be inconsistent when using different indices [305]. Whilst in my study, the Chao 1 index and ACE showed significantly lower diversity in IG compared to CG, the Shannon and Simpson index did not report significance between groups. The Chao 1and ACE diversity indices reflect microbial species richness, while Shannon and Simpson diversity indices reflect community richness and evenness [295]. However, as mentioned in 7.2.1, there is no general agreement on which diversity index is the best to use [296]. Therefore, it would be preferable if future systematic reviews and meta-analyses evaluate the findings using different indices.

7.4.2.2. Effects of relaxation intervention on microbiome diversity in the infant

Results of the analyses suggest that the use of relaxation therapy by breastfeeding mothers from 1-to 8-weeks might contribute to increased alpha diversity in their infants' gut, which was hypothesized to be beneficial for IG infants. Studies showed that higher

alpha diversity in the infant's gut reflects a more mature, adult-like community, whilst lower alpha diversity during infancy is associated with negative health outcomes later on, such as type 1 diabetes [306] and asthma [307, 308]. A study conducted in Mexican school-aged children showed a significant difference in microbiome among children in normal-weight, undernourished and obese groups ($p < 0.01$), with the normal-weight group showing greater alpha diversity than undernourished and obese groups [309]. This was consistent with a previous animal study that reported lower microbiota diversity and richness in undernourished mice [310]. Further, an RCT investigating the effects of probiotics in 40 malnourished infants reported that the use of probiotics for 50 days resulted in increased alpha diversity and increased weight in the intervention group compared to the control group [311]. These findings suggest that undernourished individuals may have decreased gut microbiome diversity compared to normal weight individuals; this is consistent with the significantly higher alpha diversity found in IG infants at 8-weeks in my study, who also had faster weight gain than CG infants. While this suggests that the relaxation therapy may have contributed to faster weight gain in IG infants by increasing the alpha diversity in their gut, it should also be considered that faster weight gain during infancy can increase the risk of later obesity and cardiovascular diseases [312]. However, studies have also shown that in preterm infants, slower early growth is associated with adverse neurodevelopmental outcomes [313]; thus, the benefits and risks of faster growth should be carefully balanced, especially for premature infants who are considered to be particularly susceptible to developmental

programming of adverse health outcomes because of their abnormal ex-utero growth patterns.

Nevertheless, several studies reported that alpha diversity was significantly higher in normal weight groups compared to obese groups either in infants [314] or in children [315, 316]. Le Chatelier et al. also suggested that higher intestinal bacterial richness was related to reduced risk of overall adiposity and insulin resistance in adults [317]. Whilst these findings suggested that the higher alpha diversity observed in IG infant in my study may be beneficial for reducing their risk of obesity, regardless of their faster early weight gain, it should be noted that most of these studies use a cross-sectional or case-control design, which can only identify a relationship between lower alpha diversity and obesity at the time of completion of the study. Hence, it is unknown if higher microbiome diversity during infancy can influence obesity risk later childhood. Given this, large cohort studies are required to investigate the early gut microbiome diversity and its influence on later weight status.

It should also be noted that higher alpha diversity is not always considered to be positive. One study reported that higher alpha diversity at 1- and 2-years of age was associated with poorer scores on the early learning (cognitive) composite, visual reception, and expressive language scales [300]. In my study, no significant differences were found in infant behaviours at 8-weeks between IG and CG, but the long-term effects on infant cognitive and behavioural development merits further investigation. Studies also suggest that alpha diversity may be higher in formula-fed infants than breast-fed infants

[318-320], despite better health outcomes in breast-fed infant [2]. Hence, the health implications of the intervention effects on increasing the alpha diversity are uncertain; it would be interesting to include later cognitive outcomes in future studies.

7.4.2.3. Transmission of the microbiome from mother to infant

Breast milk is an early source of bacteria and nutrition introduced to the infant gut within a few hours of birth; then, along with infant growth, wider environmental exposures and early intimate contact with the mother play a pivotal role in the early microbial acquisition [321-323]. In my study, the alpha diversity decreased in maternal breast milk and increased in infant gut from 1-to 8-weeks in both IG and CG. This suggests that the infant obtained a wider range of bacteria from the environment and through the intimate interaction with their caregivers over time. Feretti et al. [324] conducted source tracking analysis to estimate the contribution of the breast milk and areolar skin microbiomes to the infant gut microbiome (SourceTracker [325], version 0.9.5). Overall, after birth, mothers' stool microbiome accounted for 22.1% of the overall microbial abundance in the infant gut followed by the vagina (16.3%), the oral cavity (7.2%), and the skin (5%). Over time, the abundance of typical vaginal, oral, and cutaneous species decreased; for example, most of the vaginal species, which constituted up to 16.3% of the total abundance in the infant stool at day one, were either lost or at undetectable levels by one week after birth. This suggested that these species are likely transient inhabitants of the infant's lower gastrointestinal tract.

Interestingly, at 8-weeks, the alpha diversity was significantly lower in maternal breast milk whilst significantly greater in IG infant gut compared to the CG. This raises the question of what contributes to the additional increase of microbiome diversity in the IG infant gut. One possible explanation is that human milk oligosaccharide (HMOs) could be higher in IG mother's milk, since previous studies found that the HMOs are more abundant in healthy mothers compared to those who were distressed [326, 327]. As mentioned in Chapter 2, the HMOs are the third most abundant solid component in breast milk after lactose and lipids which can promoted intestinal colonization in the infant gut [134, 328]. Due to the budget constraints, the composition of HMOs was not assessed in my study, although this can be further investigated using the remaining samples stored at the BCH Lab. Another reason for the additional increase of microbiome diversity in IG infant's gut is that they may obtain more sources of microbiome from maternal areolar skin which was not assessed in my study. Pannaraj et al. [329] suggested that breastfed infants received 27.7% of their gut bacteria from breast milk and 10.4% from areolar skin during the first month of life. Similarly, Feretti et al. [324] showed that during the first 30 days of life, infants who were fed with more than 75% breast milk showed a 27.7±15.2% of the bacteria from breast milk and 10.3±6.0% from maternal areolar skin. All mothers in my study were asked to clean their nipples and areola with soap and sterile water, which may reduce the risk of contamination to some extent, although it would be worth collecting maternal areolar skin specimen samples in future studies to further investigate the role of areolar microbiota.

Moreover, although no statistical difference was shown between groups, it still worth noting that 11 IG and 6 CG mothers were not EBF at 8-weeks. Differences in infant gut microbiome between formula and breastfed infants have been identified in several studies [320, 330, 331]. An experimental study examined the gut microbiome in 91 term infants and showed that the *Bifidobacterium* and *Bacteroides* were significantly higher, while *Streptococcus* and *Enterococcus* were significantly lower in breast-fed versus formula-fed infants [320]. Another study investigated the gut microbiome in 102 term infants with EBF, mixed feeding, and exclusive formula feeding [332]; results showed a significant association between microbial community composition and feeding method ($P = 0.01$; $Q < 0.001$). Similar results were found in moderate to late preterm infants [333]. Moreover, some studies found that the alpha diversity in formula-fed infants is higher than in breast-fed infants[318-320]. It can be hypothesised that the non-significantly higher rate of mixed feeding in IG infants resulted in the greater alpha diversity in comparison to the CG. The small sample size for microbiome analysis might prevent the detection of significant difference in feeding method between groups in my study.

7.4.3. Changes of microbiome from 1-to 8-weeks

From 1-to 8-weeks, the alpha diversity slightly decreased in breast milk and infant gut in both IG and CG, but no significant changes were observed. The beta diversity of breast milk and infant stool samples at 8-weeks was significantly different compared to 1-week samples in the CG, but not the IG. On the one hand, this suggests that the relaxation

intervention might have no effect on beta diversity from 1- to 8-weeks. However, it could also be hypothesised that the relaxation intervention might prevent the changes in beta-diversity in breast milk and infant's gut which would normally be seen from 1-to 8-weeks, which may result in higher consistency of the microbiome community structures in IG breast milk and infant gut. However, based on current data, no supporting evidence was available to identify if the impact of relaxation therapy on beta-diversity is good or not. As discussed in previous sections, gut microbiome diversity may be associated with both growth and cognitive outcomes, hence, longer-term follow-up recording anthropometry and developmental outcomes should be incorporated in future studies. Also, further annotated sequence analysis will be conducted after the whole data released, which can identify the exact species changes in CG samples at Family and Genus level, by which the impact of such changes could be investigated.

Moreover, from 1-to 8-weeks, significant decreases in *Firmicutes* and increases in *Proteobacteria* were observed in CG breast milk samples and, although not statistically significant, the decrease of *Firmicutes* in CG infant gut was more obvious than in the IG. This implies that breast milk might be the main sources of *Firmicutes* for infant gut colonization. Several studies have examined the *Firmicutes* at phylum level and its relationship with childhood obesity. A systematic review appraised seven studies on the relationship between gut microbiome and childhood obesity; results showed that *Firmicutes* may be positively correlated with weight gain [334]. However, it should be mentioned that four studies in this review are cross-sectional studies which could not

identify the relationship between microbiome diversity and weight gain from a longitudinal perspective. While more cohort studies are needed, current evidence may suggest that the relaxation therapy in my study could have prevented the decrease in *Firmicutes* in breastmilk over time, which contributed to the higher infant weight in the IG at 8-weeks compared to the CG.

Further, animal studies reported that diets with higher sugar and fat may be associated with an overgrowth of *Firmicutes* in mice [335, 336]. Two studies examined the gut microbiome in lean and obese mice and reported that the gut microbiota can affect energy balance by influencing the efficiency of calorie harvest from the diet [337, 338]. In chapter 6, I reported that the fat content of breast milk was non-significantly higher in IG mothers. It might be hypothesised that this higher fat content nourished the *Firmicutes* in IG mother's milk and prevented it from reducing as seen in the CG. This in turn could have resulted in a greater transfer of *Firmicutes* to the infant's gut which contributed to faster weight gain. However, it should be noted that although the reduction of *Firmicutes* appeared to be less in the gut of IG infants, this was not significant. Further investigations in larger samples are needed to confirm if the relaxation intervention could directly influence the abundance of *Firmicutes* in the infant's gut and its relationship with both breast milk composition and infant weight gain.

In addition, studies have reported that the abundance of *Proteobacteria* can be associated with obesity [309, 334, 339]. In the Mexican study mentioned above, *Proteobacteria* gradually increased from undernourished children (3.1%) to normal

weight children (7.8%) and then to the obese children (15.1%); the difference between obese and undernourished children was significant (p=0.002). Similar findings were shown in Zhu et al. [340] who analysed 63 child stool samples and found that the abundance of *Proteobacteria* was significantly higher in the obese group than in healthy controls. Given these findings, is can be suggested that the relaxation intervention might prevent a significant increase of *Proteobacteria* in breast milk which could lead to higher abundance in the infant's gut through breastfeeding, increasing the risk of subsequent childhood obesity.

Overall, the findings from my microbiome analyses are consistent with my hypothesis that the microbiome could be one of the mechanisms underlying mother and infant signalling. However, it should be mentioned that the microbiome examined in breast milk might also contain species from maternal areola skin or from the infant's mouth. As mentioned in chapter 2, there are three routes that are suggested to be the origins of the breast milk microbiota: maternal areolar skin, retrograde flow from the infant's oral cavity, and movement of microbiota from the maternal gut to the mammary gland [139, 144]. Although all mothers were asked to clean their nipples and areola with soap and sterile water to reduce contamination, the sample collection was conducted by mothers with no supervision due to privacy considerations. As suggested by Pannaraj et al. [329], *Proteobacteria* are the dominant phylum in milk, whilst *Firmicutes* are dominant in areolar skin. According to this, if the breast milk samples contained species

from the areola skin, the composition of *Proteobacteria* and *Firmicutes* might be affected.

7.4.4. Comparisons of the data from my study with other infant studies

It has been suggested that the gut microbiome composition in healthy new-borns shows major longitudinal changes during the first three years after birth until it settles into an adult-like anaerobic pattern [341, 342]. During the first year of life, the infant gut microbiome changes dynamically in response to early environmental exposures [343]. Kuang et al. [344] compared the richness and evenness of the faecal microbial communities between neonates (1-4 days after birth) and ~2-month-old healthy term Chinese infants. The alpha diversity was significantly higher in the gut at 2 months compared to neonates ($p < 0.01$), which was not observed in my study. However, in my study, significant compositional differences (beta diversity) were identified in CG infants between 1-and 8-weeks of age, which was consistent with Kuang et al. Moreover, Kuang et al. reported the most dominant bacteria in the neonates' gut at phylum level, which were *Proteobacteria* (mean relative abundance = 52.3%), *Firmicutes* (40.2%), *Bacteroidetes* (6.1%) and *Actinobacteria* (0.9%); compared with the neonates, the infants at 2-months had slight decreases in the abundance of *Proteobacteria* (43% vs. 61%), and increases in *Firmicutes* (47% vs. 35%), but these changes were not significant [344]. In my study, contrasting results were observed, where the *Proteobacteria* in the infants' gut increased from 1- to 8-weeks whilst the *Firmicutes* decreased, although the changes were again not significant. One explanation could be the different gestational

age and delivery mode between my study population and that in Kuang et al, which included term infants born both vaginally and by caesarean section, whilst in my study only vaginally delivered late preterm and early term infants were included. As suggested by a number of studies, the gestational age and delivery mode are factors associated with the infant's gut microbiome composition [341-343]. Moreover, it should be mentioned that the microbiome was not compared in the same individuals in Kuang et al.; the study included a small group of infants aged 1-4 days (n=15) and another at 2-months of age (n =14).

More recently, an RCT assessed the effects of probiotics on faecal microbiota composition of 227 Chinese moderate to late preterm infants (GA=32-35 weeks) from day ten to 4-months corrected-age [345]. A significantly greater alpha diversity in infant gut was observed at 4-months compared to day ten, which is consistent with Kuang et al. In addition, the abundance of four major gut phyla—*Firmicutes*, *Actinobacteria*, *Proteobacteria*, and *Bacteroidetes*—changed significantly between day ten and 4-months. However, the authors did not provide the direction of changes, instead, they highlighted that the changes in bacteria from the Family level were complex (eight taxa from the phylum *Firmicutes*, such as *Lactobacillaceae*, increased while the *Staphylococcaceae* decreased from day 10 to 4-months). However, since I currently do not have data available from my study at Family level, it is difficult to make comparisons with this study.

A longitudinal study of 107 American mother-infant pairs showed that *Proteobacteria* and *Actinobacteria* accounted for more than 50% of the community in the infant gut after birth; moreover, the alpha diversity significantly increased in infant gut but not breast milk ($p = 0.06$) with infant age, which was similar to my study. Additionally, this American study also reported significantly increased beta diversity (based on unweighted UniFrac) in breast milk and a steady decrease ($p < .001$) in infant gut during the first 6 months[329], which was also observed in the control group in my study.

Studies have shown that different social structures may influence the extent of vertical transmission of microbiota from mother to infant, and the flow of microbes and microbial genes among members of a household. For example, different cultural influences such as exposure to pets and livestock could influence how and from where the gut microbiota/microbiome is acquired [344]. Kuang et al. [344] compared the gut microbiota composition in Chinese infants with that of infants from five different countries. A total of 206 infant stool samples were analysed. At the phylum level, significant differences in gut microbiome composition were observed among the six countries. All the Chinese infants and 70% of Brazilian infants showed highly abundant *Proteobacteria*, whereas 82% of American infants, 54% of Swedish infants and 79% of Canadian infants presented highly abundant *Actinobacteria*. Most infant stools from Bangladesh (70%) and 33% from Sweden were highly abundant in *Firmicutes*. Multivariate analysis of variance was applied to evaluate the contributions of different factors to the variations in the infant gut microbiota; the country of residence accounted

for most (19.6%, $p < 0.001$) of the variation whilst the age, delivery mode and feeding pattern accounted for only 0.42%, 0.46%, and 0.09% ($p > 0.1$) of the variation respectively. These differences in microbiome composition between countries suggests that cultural or geographical factors may have a strong impact on the composition of gut microbiota during early infancy.

7.4.5. Strength and limitations

This is the first study to investigate whether the microbiome could act as a signal between mother and infant during breastfeeding using an experimental approach. By altering the maternal psychological status using the relaxation intervention, this is also the first RCT to evaluate the impact of maternal psychological status on microbiome composition in BM, MS, and IS. The sample size is reasonable to detect significance between assessed groups. However, there are certain limitations in this study. Firstly, I did not collect specimen samples from the mouth of mother or infant, maternal areolar skin or vagina, which may have contributed additional bacteria to infants. Secondly, considering privacy, most samples were collected by mothers without supervision, and although clear instructions were provided in advance, this might have led to contamination of the collected samples. For example, it is not clear whether the breast milk samples may have been contaminated by skin microbiota although all mothers had been asked to clean their nipples and areola before collection. Moreover, it is not clear if the infant stool samples collected from the diaper were contaminated by their urine, although advice about how to avoid collecting urine was provided in advance. Thirdly,

as mentioned in chapter 2, the HMOs in breast milk may promote the persistence of specific bacterial lineages. Due to the budget for this project the HMOs were not assessed, although there are remaining samples stored at the BCH Lab which can be analysed in future. Last but not least, results provided in this thesis were limited by the incomplete release of data by [3] Novogene, and further investigation will be conducted in the future when the full data are released.

7.5. Conclusion

Overall, these preliminary findings from my microbiome analyses are consistent with my hypothesis that the microbiome could be one of the mechanisms underlying mother and infant signalling. Results of the microbiome analysis suggested that compared to control mothers, mothers who used the relaxation therapy from 1-week to 8-weeks had lower alpha diversity in gut microbiome; this could be a result of reduced maternal stress, and might in turn could lead to a further reduction in maternal stress at 8-weeks. The alpha diversity in breast milk was also significantly lower in IG than CG, consistent with the transmission of microbiota with lower alpha diversity from IG mother's gut to their breast milk through entero-mammary trafficking. The microbiome is then transmitted to the infant's gut through breastfeeding, although at 8-weeks, the IG infants showed significantly higher alpha diversity than the CG, which was inconsistent with the effect seen in maternal gut and breast milk. It is possible that the HMOs, which contribute to

[3] * There was a Covid-related issue which is described in the separate Covid impact statement

intestinal colonization during infancy, might be increased in IG mother's breast milk due to their decreased stress. The composition of HMO was not assessed in my study, but this can be further investigated in future studies. The increased alpha diversity in the gut of IG infants at 8-weeks, which may be caused by the relaxation intervention as outlined above, could be part of the mechanism underlying the increased weight gain seen in relaxation group infants. Moreover, significantly different beta diversity was observed in maternal stool between IG and CG at 8-weeks but not 1-week, suggesting the mothers in IG presented higher similarity than CG regarding their gut microbiome at 8-weeks. Further analysis on microbiome differences at different classification levels will be conducted when the whole dataset is released.

Chapter 8

General Discussion and Conclusions

This chapter provides a summary of the findings of the pilot study and the main RCT, followed by an overview of the findings in the context of potential mechanisms of signalling, including physiological, psychological and anthropological perspectives, as well as the potential role of the microbiome in mother-infant signalling. Moreover, strengths and weaknesses of the whole PhD project are outlined, followed by the research contributions considering scientific, clinical, and public health aspects of breastfeeding and infant growth. Finally, suggestions for future research are provided. This chapter ends with a conclusion of my PhD project -- the BABY study.

8.1. Summary of the findings

8.1.1. Overview of the pilot study

In the pilot study, a randomised trial using within-subject design was conducted to compare the effects of relaxation meditation tape (RM), music tape (M), relaxation lighting (L), combined RM+L, and combined M+L on perceived and physical relaxation. Considering the aim of the pilot study was to identify the most suitable relaxation intervention to be used in the main RCT, the study sample was selected to be close to that planned for the main RCT, which was healthy Chinese primiparous mothers (aged 23-45 years) who were breastfeeding their infants within 2 years after delivery.

Results of the pilot study showed that compared to the control state, all five relaxation interventions had significant effects on increasing the fingertip temperature (FT) and perceived relaxation. When compared to the pre-test state of each treatment, the RM, RM+RL, M+RL produced a significant reduction in blood pressure (BP) and heart rate (HR); moreover, the FT and perceived relaxation were significantly increased after each of these three treatments.

When compared to the control state, the RM resulted in the greatest mean difference from pre to post test in SBP, DBP and HR, while the M+RL produced the greatest mean difference in FT and perceived relaxation. The RM also produced significant changes in all measured outcomes (BP, HR, FT, and perceived relaxation) in comparison to the control state. Notably, four mothers (with infants aged 1-3 months) experienced milk

let-down (ejection) during the RM treatment, but not in response to other treatments or during the control session. This observation suggested that this intervention could be particularly effective in breastfeeding women.

The pilot study was the first to evaluate several relaxations approaches together in breastfeeding mothers using an experimental design. Results of the pilot study not only confirmed the relaxation meditation as a simple relaxation technique showing the best effects on improving physical and perceived relaxation in Chinese breastfeeding mothers, but also identified that the different relaxation techniques might influence different components of an individual's stress response. Hence, physiological and perceived indicators may detect a reduction in physiological and cognitive stress respectively, which means a mother might "perceive" herself as relaxed but her biological indicators such as BP, HR, might not be consistent with her perceived results, and vice versa. However, based on current results, it is still unclear which relaxation type is more relevant in the context of lactation. Therefore, further investigation is suggested on whether physiological or psychological relaxation is more strongly related to better breastfeeding outcomes.

8.1.2. Overview of the main study

The population included in the main RCT were healthy Chinese primiparous mothers who were planning to breastfeed their LP and ET infants (GA 34-37 weeks) for at least 8-weeks. As discussed in Chapter 2, there were two reasons for choosing this population.

Firstly, the breastfeeding rates in LP and ET mothers are lower than in term and post-term mothers [346], suggesting the need for more research in this population. Secondly, the higher risk of breastfeeding difficulties and the relatively poorer health in LP and ET infants may result in higher maternal stress and greater mother-infant conflict. As suggested in previous studies, LP infants are more likely to experience unsuccessful establishment of lactation and are especially susceptible to early breastfeeding failure [46-48], including early breastfeeding cessation, parental feeding-related anxiety, and re-hospitalization for feeding issues [49]. Moreover, infants born at 37 weeks have a higher risk of respiratory distress syndrome (RDS) compared to other full-term infants. Given these factors, mothers may experience more stress during the postpartum period and have to make trade-off decisions on whether to invest more energy on breastfeeding or her own recovery. In this context, the relaxation therapy was hypothesized to be more effective in this population. Consistent with the primary hypothesis, listening to the relaxation tape from 1- to 8-weeks postpartum resulted in a reduction in maternal stress and greater infant weight gain; and the effect on weight gain was greater in girls than in boys.

Moreover, a non-significant effect on length gain was detected in IG infants. Dose-response analysis showed that greater use of the tape (total and average times, as well as the total days of usage) was correlated with greater reduction of maternal stress. No significant differences were found in maternal anxiety between IG and CG.

Inconsistent with the secondary hypothesis, the relaxation therapy did not show significant effects on breast milk fat, carbohydrate, protein, and energy content, however, a non-significant trend towards higher breast milk fat and energy content was observed in IG mothers. The mean value of the estimated milk intake was not significantly different between groups. No significant effects were shown on maternal breastfeeding attitudes or infant eating behaviours (total BEBQ scores nor any categories of the BEBQ). Moreover, mothers who received the relaxation therapy did not report significantly different durations of any assessed infant behaviours compared to control mothers. However, reduced maternal stress was significantly associated with longer awake/happy duration and more frequent awake (happy) periods in the whole study cohort.

Microbiome analysis showed that mothers who used the relaxation therapy from 1-week to 8-weeks had lower alpha diversity in gut microbiome (examined using stool samples) compared to control mothers. The alpha diversity in breast milk was also significantly lower in IG than CG. In contrast, the IG infants showed significantly higher alpha diversity than the CG. Significantly different beta diversity was observed in maternal gut microbiome between IG and CG at 8-weeks but not at 1-week. Moreover, from 1-to 8-weeks, significant decreases in *Firmicutes* and increases in *Proteobacteria* were observed in CG breast milk samples, but not in IG samples.

8.2. Interpretation of the Findings

8.2.1. Physiological and psychological factors in mother-infant signalling

Figure 8.1 outlines a summary of the findings in the context of proposed pathways of signalling in the pilot and the main RCT, and **Figure 8.2** summarises the findings of the research from both biological and anthropological perspectives. Both physical and psychological factors could result in increased infant growth via increased nutrient intake and/or non-nutrient factors such as hormones and microbiome, as well as reduced energy expenditure as a result of a longer duration of sleeping and/or shorter duration of distress in the infant. Overall, results of the pilot study and the main RCT indicated that the relaxation therapy was effective in improving relaxation and reducing stress in breastfeeding mothers. In the main RCT, the non-significant trend of increasing fat and energy in breast milk supported that the relaxation therapy may favourably affect breast milk composition and thereby positively influence infant weight and length gain. This could be a potential mechanism for the physiological signalling between mother and infants. In terms of psychological signalling, theoretically, infant growth can be improved by promoting behaviours such as increased sleeping and reduced crying, since those behaviours could help to reduce energy expenditure. It has been suggested that by reducing maternal stress, the mother-infant interaction or bonding might be strengthened, which could improve sleeping quality in both mother and infant [347, 348]. Infant sleeping behaviour has been demonstrated to have an impact on infant growth due to the reduced energy expenditure, especially during the first four to six

months of infancy life when energy requirements for growth are greatest [349, 350]. However, in my study, the reported infant behaviours were not influenced by the intervention, although IG infants had higher mean values for sleeping and lower mean values of "distress" behaviours; as discussed in Chapter 6, these analyses were limited by the small number of participants who returned the 3-day behaviour questionnaire which reduced the power to detect effects. In the MOM study, longer sleep duration was observed in IG mothers at 6-weeks. Considering the measurement for infant behaviours in my study was the same as in the MOM study, this inconsistency might be due to the cultural differences between populations. For example, the MOM study reported that most of the involved mothers were co-sleeping with their infants, whereas the prevalence of co-sleeping in Chinese families was reported to be 46% to 60% from birth to eight months [351]; mothers of younger infants were less likely to co-sleep since they prioritised the need for better sleep to recover from the delivery. Although the mother's sleeping pattern was not assessed in my study, during the data collection period, some mothers shared their experiences with me that during their confinement period they would prepare expressed breast milk so their husband or a nanny could feed the baby at night. Given this, in my study the relaxation intervention may have had less potential to influence infant sleeping behaviour if mother and infant were sleeping separately; moreover, the mother might even have provided inaccurate data about the infant's night sleeping if their husband or nanny had fed the infant during this period.

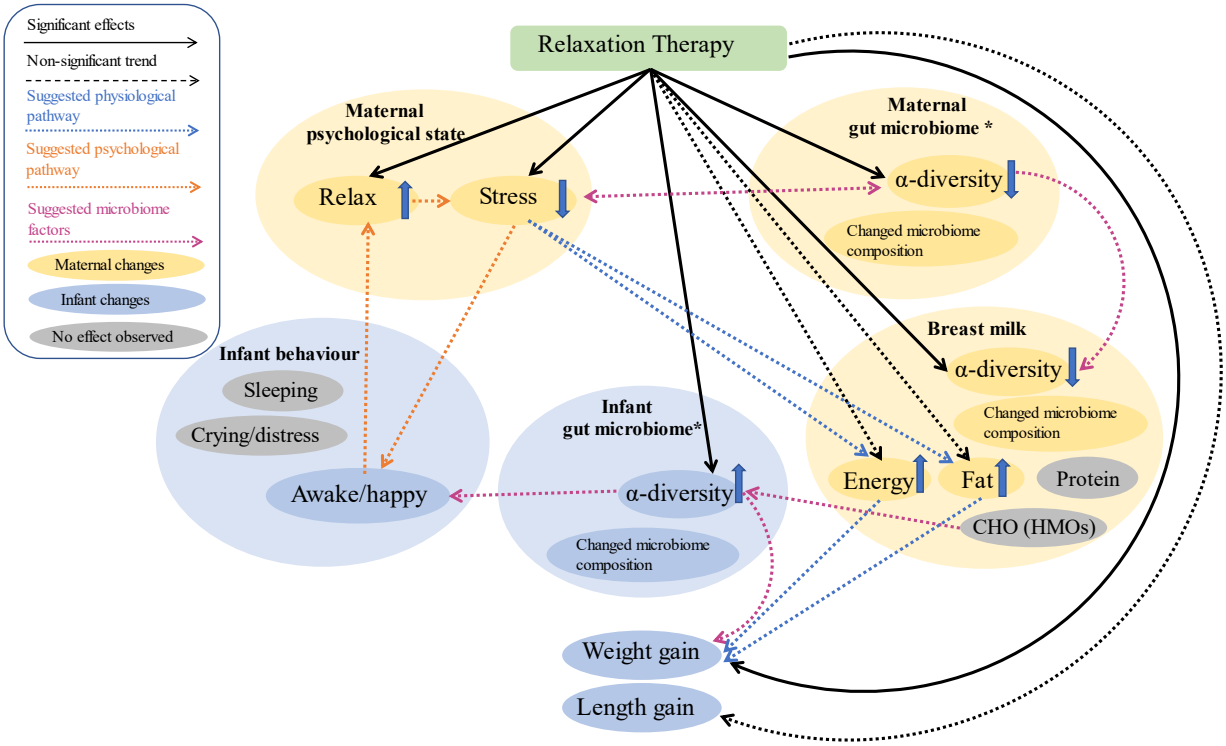


Figure 8.1. Overview of the observed effects of the intervention and the suggested pathways of mother-infant signalling.

Notes: CHO=carbohydrates; HMOs=human milk oligosaccharides.

* Gut microbiome was examined using stool samples. The HMOs in breast milk were not examined in the current research due to the budget; however, it is suggested to be a potential factor that is involved in mother-infant signalling. Future investigation will be conducted using the stored milk samples from the current research to confirm this hypothesis.

Results from the observational analyses showed that reduced maternal stress was significantly associated with longer awake/happy duration and more frequent awake and happy periods when assessing all subjects, although no significant differences were found between IG and CG. As discussed in Chapter 6, this may be due to the fact that relaxed mothers are more receptive to their babies' facial expression compared to mothers who are stressed. Studies showed that mothers with postnatal anxiety or depression have difficulties in understanding the infant's behavioural cues and find it difficult to respond appropriately to their infants [284-286]. Hence, mothers who were more relaxed may have been more sensitive to their infant's cues, which could have been reflected in their diary entries and resulted in the significant association between maternal stress and infant awake and happy duration and frequency. In addition, by receiving the infant's cue that they are awake and happy, mothers might be further relaxed, and consequently produce more milk with higher energy, which may result in increased weight gain. However, this interpretation should be treated with caution since the observational analyses cannot demonstrate a causal relationship between maternal stress and infant awake and happy duration. In the main RCT, both duration and frequency of the awake/happy behaviours were not significantly different between IG and CG; as previously discussed, this might be due to the reduced sample in this assessment which limited the power to detect intervention effects. Hence, the cause-effect relationships between reduced maternal stress and the awake/happy behaviour should be further investigated in larger samples. Detailed suggestions for future research are provided in section 8.6.

Considering the generalisability of my findings to other populations, it should also be acknowledged that the signalling pathways may work differently in different populations, since human beings exhibit enormous behavioural diversity, both within and between populations [352]. Therefore, the stress resulting from environmental factors might be different in other populations compared to Chinese mothers. For example, a large majority of mothers in Asian countries practice the traditional confinement period after birth; their behavioural characteristics, postpartum psychological status, and the interaction between mother and infants can thus be different from mothers in western countries [352]. Moreover, even within Asian countries, the confinement practices are varied. A study compared the ethnic group differences regarding confinement practices among Chinese, Malay, and Indian mothers and reported that Chinese mothers showered less and were more likely to depend on confinement nannies during this period than mothers from Malay and Indian ($p < 0.001$ for all), whilst Malay mothers tended to make greater use of massage therapy ($p < 0.001$) [353]. In addition, whilst the MOM study suggested that the confinement period may reduce the potential for stress and anxiety in Malay mothers, a systematic review in Chinese population indicated that the confinement practice may either contribute to or fail to protect against postpartum depression, due to the generally diminished social support in contemporary society, conflict with the mother-in-law and the tension experienced by modern women as they work to balance traditional with contemporary values. Hence, the diversity in culture, ecology and maternal behavioural characteristics could contribute to the behavioural differences within and/or between populations. In

this context, the relaxation intervention might have different effects in different populations, and mothers in my study might either experience different levels or types of stress or respond differently compared to those in other studies.

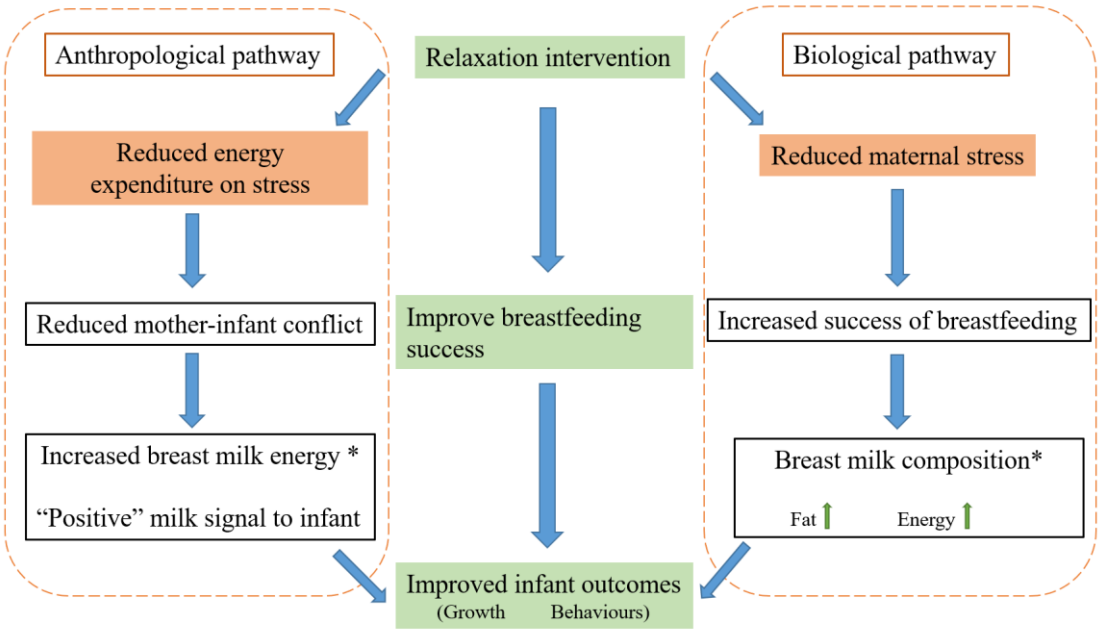


Figure 8.2. Potential pathways of the mother-infant signalling interpreted from biological and anthropological perspectives.

Note: * non-significant trend (p=0.07)

8.2.2. Anthropological perspective

As discussed in Chapter 2, lactation is costly and the maternal energy budget could be influenced by increased tension based on Trivers' parent-offspring conflict theory, that the offspring is selected to demand more resources than the mother is selected to provide; this conflict starts from foetal life, and is expected to increase during the postpartum period, since this period can be highly energetically demanding as mothers need to recover from delivery and feed their offspring at the same time [354-356].

From the anthropological perspective, there are several potential mechanisms by which mother-offspring conflict may influence the maternal investment strategy during lactation (**Figure 8.2**). There are two possible pathways for the maternal investment strategy which could be affected by the intervention. Firstly, by reducing maternal stress during the postpartum period, the maternal energy budget for reproduction could be increased. The mother's trade-off decisions may then be altered to invest in the infant by providing more energy in breast milk; this may be supported by the findings of a non-significant trend towards increased breast milk fat and energy in IG mothers, potentially increasing energy intake and explaining the greater weight gain in these infants compared to CG infants. Secondly, if the infant receives more energy from breast milk, they might consequently have a reduced need to vocalise hunger (crying behaviour) and potentially have longer periods awake (happy). This is consistent with the observational results of my study which found that lower maternal stress at 8-weeks was significantly associated with longer duration and more frequent awake and happy episodes per day. Both animal and human studies showed that lower consumption of milk energy or volume could result in higher vocalisation or demand by the infants [357, 358]. However, it should be mentioned that the infant's awake and happy behaviour was not significantly different between IG and CG, hence my findings could not confirm a causal relationship between the effects of relaxation intervention and infant behaviours.

Moreover, the energy requirement was suggested to be different between genders during early infancy. Previous studies showed that male infants could be more energy

costly since they are more vulnerable than females to suffering complications, such as respiratory distress syndrome [359] and neonatal anaemia [360], particularly in low birthweight infants [359, 361]. This has been previously recognised as “male disadvantage” which describes the sex-bias observed in males regarding perinatal outcomes [362]. Hence, optimising the early life nutritional could be important to minimise the “male disadvantage”, which might lead to a potential parental bias towards male offspring. Trivers hypothesised that if environmental conditions are stressful, mothers would gain greater fitness returns if they invested in daughters compared to sons [363]. In this context, the mothers in my study can be seen as not in good fitness condition due to their LP and ET births, so their investment strategy may therefore be biased towards girls, which could potentially explain the higher milk energy and fat content in breast milk of IG mothers of girls. This sex-specific investment may ultimately influence infant growth trajectories during the postpartum period [364].

On the contrary, some animal studies have suggested that mothers might invest more in female offspring, potentially because their maturation rate is faster than males, which could enable the next generation to start reproducing earlier [365, 366]. However, some human studies did not find significant gender-specific differences in breast milk composition [367-369]. Also, in the MOM study, no significant gender differences were found in breastmilk composition and infant growth rate. Inconsistent with most human studies, my findings showed that the effects of relaxation therapy were different by gender, with a greater effect of the intervention on weight gain in girls than boys.

Mothers of girls also showed higher milk energy (p=0.046) and potentially higher fat content (p=0.07) at 8-weeks in the IG. One the one hand, my results suggest that the relaxation intervention might alter maternal investment in girls more than boys. On the other hand, as discussed in Chapter 6, compared to mothers of boys in the IG, mothers of girls spent significantly more days listening to the relaxation tape, hence the greater beneficial effects on weight gain in girls might reflect greater exposure to the intervention. Hence, mothers of girls may have been more relaxed and the let-down reflex may have been more efficient, leading to a higher hind milk intake [18, 266].

It is possible that by tailoring nutrition during early life health and development could be optimised in infants of both sexes. However, available evidence is inconclusive regarding Trivers' theory that maternal investment would be different by gender, and if that in turn is related to different growth rates between genders. Additionally, the inconsistent results from current human studies may be due to demographic differences among study populations and the different unstandardised methods of milk sampling. Therefore, future studies investigating gender effects on breastmilk composition and intake in large samples are recommended.

8.2.3. Microbiome factors

As shown in **Figure 8.1**, it was hypothesised that the microbiome could be involved in mother-infant signalling. Findings of my analysis showed that by using the relaxation therapy, the microbiome diversity in IG in the mother's gut was significantly lower

compared to that of the CG, which could be related to better mental health (reduced maternal stress) as discussed in Chapter 7. On the other hand, reduced maternal stress can also result in decreased alpha diversity [132, 140]. There could therefore be a bidirectional relationship between maternal stress and the maternal gut microbiome diversity as shown in **Figure 8.1**. Consistent with the maternal gut microbiome, the alpha diversity in maternal breast milk was also significantly lower in IG compared to CG, possibly reflecting the transmission of microbiome from maternal gut to breast milk though the EMT. The microbiome could then be transmitted to the infants' gut through breastfeeding. As shown in my results, the microbiome diversity in IG infant's gut was significantly higher than in that of the CG, which was suggested to be related to greater weight gain [309, 311]. These preliminary findings are consistent with a role of the microbiome as a signal between mother and infant though breastfeeding which may have impact on infant growth.

Although the composition of HMOs in breast milk was not assessed in my study due to budget constraints, it is hypothesised to be another influencing factor in mother-infant-signalling, which plays a role in the infant's developing intestinal colonisation. Previous studies reported that the HMOs are more abundant in healthy mothers compared to those who were distressed [326, 327]. Accordingly, it can be hypothesised that the IG mother's breast milk may contain more HMOs than CG's, which may have contributed to greater alpha diversity in the IG infant's gut. This hypothesis can be further investigated using the remaining samples stored at the BCH Lab.

Moreover, results of the microbiome analysis also showed the relaxation therapy can influence the change in microbiome composition from 1 to 8 week. However, current data cannot provide more evidence on exactly what changes have occurred. This can be further investigated after the whole dataset is released.

8.3. General Strengths of the Research

A summary of the general strengths of my study are as follows:

- The design of the pilot study allowed the selection of the most suitable relaxation intervention for the main RCT, whilst the within-subject design allowed several relaxation interventions to be tested and compared in a single study, which provided evidence for future intervention studies in breastfeeding women.
- The randomised controlled study design allowed the detection of causal relationships between the intervention (relaxation meditation tape) and maternal psychological state, breast milk composition, infant growth and behaviours.
- This was the first RCT which assessed relaxation therapy in breastfeeding mothers and their LP and ET infants with a reasonably large sample size (n=96). The original planned sample size was 120 with allowance for potential drop-outs or failed measurements, but the actual compliance rate was very high, hence, as calculated in Chapter 4, 82 mother-infant pairs (41 per randomised group) allowed the detection of the pre-specified effect size between groups that I was aiming to detect at 80% power with a significance level of 0.05.

- Compared to previous RCTs in mothers of preterm infants, my study provided a comprehensive evaluation about the mother-infant signalling from psychological, psychological, and anthropological perspectives.
- Mothers were blinded to the use of relaxation therapy; they were told that the aim of the study was to investigate maternal breastfeeding outcomes following late preterm/early term delivery. This may have reduced the risk that mothers in the control group would seek alternative interventions to relax.
- The reasonably long intervention period enabled me to investigate the effects of longer periods of relaxation therapy – considering that many previous studies have only investigated a single relaxation session or a short period of use (mostly less than 14 days).
- Stringent pre-study training and frequent validation of instruments and procedures, could maximise the objectivity of the study and strengthen the internal validity.
- There was a high compliance rate for most of the outcomes: all mothers provided data on PSS and BAI, and weight and length were obtained from all infants. More than 90% mothers provided breast milk samples for milk composition analysis.
- This was the first RCT to investigate the role of the microbiome in mother-infant signalling following LP and ET delivery. Although the whole dataset is not yet available, current findings showed that by manipulating the maternal psychological status using the relaxation intervention, the alpha diversity of microbiome in breast milk, maternal and infant's gut was altered; the intervention also influenced the change in microbiome from 1 to 8 week.

- Most outcome measurements in the main RCT were the same as in the MOM study, which made the findings from these two studies comparable.

8.4. Limitations of the Research

Limitations of the whole study have been discussed in the corresponding chapters throughout the thesis. A summary of the limitations is as follows:

- Single blind design. However, due to the nature of this relaxation intervention, it was not possible to blind the subjects or researchers to the intervention. Potential detection bias might have occurred, for example, whilst infant weight was measured directly by the digital scale and less susceptible to bias, the measurement of length was based on a more subjective reading; it might be biased if the research nurses overestimated the length of IG infants. However, since the research nurses had no particular interest or investment in the results of the study, the risk of detection bias was considered to be low.
- Another bias could be the self-reported questionnaires provided by mothers. Although mothers were blinded to the randomisation, mothers in the relaxation group may potentially have expected to be “relaxed” after the intervention, which might have biased some of their responses. Their report on their infant’s behaviour might also be biased if IG mother consider the infants could benefit from the intervention. Thus, more objective assessments for those variables are suggested in

future together with self-reported questionnaires, such as a 24-hour heart rate recording watch for the measurement of maternal stress.

- The RCT involved several research assistants for data collection. It was impossible to be sure that the standard operating procedures for infant measurements and milk sample collection were followed consistently. However, I tried to come with the nurses for most of the home visits, and regular training was arranged every month to enhance the standard operating procedures.
- Biological sample collection: breast milk samples were collected by mothers with a hand pump provided by us. Most of those mothers did not want to pump their milk in front of others. Potential bias may occur if the mother did not collect fore-milk as requested. However, due to the randomised study design, the variation in milk composition should apply equally to both IG and CG and would not necessarily affect the comparison between groups.
- Stool sample collection: the stool samples were collected by mother themselves with detailed instruction provided. The variation in stool sample collection should apply equally to both IG and CG due to the randomised controlled design. However, only vaginally delivered mothers were involved in the analysis and the reduced sample size might affect the statistical results, although the populations in the microbiome analyses were balanced for baseline characteristics.
- Due to the influence of COVID-19, the whole data of the microbiome analyses were not released yet.

- Due to the ethical application difficulty in preterm infants in China, only test-weighing could be used in my study for milk intake assessment. However, the compliance rate for this assessment was low and this limited the analyses of breast milk intake.
- Selection bias during the recruitment: the education level was higher than general Chinese population. It seems that mothers with higher education were more interested in participating. Due to this, the results could not be generalised to the whole population in China.

8.5. Contribution of the Research

Results of my research confirmed the effects of relaxation intervention on reducing maternal postpartum stress and improving infant weight gain, and increasing the microbiome diversity in the infant's gut. My study sample involved late preterm and early term infants who are a vulnerable and understudied group of infants in whom mother-infant conflict is likely to be greater. My research findings have several implications for both clinical practices and anthropological research. These are discussed in the following sections.

8.5.1. Implications for the early hospital management of LP/ET mother and infants

In clinical settings, mothers of LP infants may face more challenges than ET mothers, especially when their baby is transferred to the NICU and the skin-to-skin contact is consequently delayed. Indeed, in my study, whilst all ET mothers had skin-to-skin contact within the first hour, 11 of the 57 LP mothers (19.3%) did not. Previous studies

suggested that early skin-to-skin contact was associated with successful early initiation of breastfeeding [244]. Consistently, in my study, the initiation of breastfeeding in LP mothers started significantly later than in ET mothers. Based on WHO recommendations, the practice of skin-to-skin contact should start within one hour after birth; health care providers should encourage women to recognise when their babies are ready to breastfeed and offer help if needed [238]. Most clinical settings in Beijing encourage the skin-to-skin contact to be undertaken immediately after birth for all mothers with healthy infants. For mothers with LP or ET infants, if the infant is generally healthy (without severe malformations and syndromic disorder or needing resuscitation after birth), skin-to-skin contact is also recommended after birth. Moreover, most hospitals in Beijing suggested using mother's expressed breast milk to ensure breastfeeding. Mothers of hospitalized preterm infants are recommended to initiate lactation through milk expression routinely and provide expressed breast milk to their infants[370]. However, during the infant's hospitalisation, concern about the baby's health can expose mothers to stress, which may inhibit the let-down reflex [114, 371] and influence the milk flow. Besides, hand/pump expression are not as effective in stimulating the milk supply as suckling an infant at the breast [372]. Consequently, successful breastfeeding can be challenging among mothers of hospitalised infants. In this context, the use of the relaxation meditation could be effective for reducing maternal stress, and consequently promoting breastfeeding; although in my study, the effect of relaxation therapy was not tested in clinical settings, it is worth considering whether this approach could be used as a simple and non-invasive tool for mothers who have difficulty establishing

breastfeeding in hospital, especially for LP and ET mothers who may experience mother-infant separation if their infants are transferred to the NICU due to health issues.

8.5.2. Implications for the care of LP/ET mother and infants after discharge

Findings of my study highlighted the importance of minimising maternal stress after discharge from the hospital, which could reduce the tension between mother and infant by reducing maternal energy expenditure on stress, allowing mothers to invest more in their infant. The relaxation meditation tape as a simple and cost-effective technique can easily be offered to mothers who are facing postpartum stress during their confinement period. Compared to previous studies, the relaxation meditation in my study was saved and used on the mother's mobile phone, which increased convenience. However, further investigations are needed to confirm the effect of the relaxation intervention in different mothers (eg., mothers of low-birth-weight infants, mothers of twins) and on different infant outcomes (eg., body composition and cortisol level); the longer-term effects of relaxation therapy also merit further investigation, and future intervention studies with longer follow-up periods are suggested.

Moreover, my results showed that maternal compliance with the use of relaxation tape was higher in mother of girls than boys, suggesting mothers of girls might have higher stress during the postpartum period than mothers of boys and consequently be more likely to seek relaxation therapy. However, although mothers of boys may not be so aware of the stress, mother-infant conflict exists in mothers of both boys and girls; this

suggests that if the relaxation therapy is used in clinical practice, the nurse may need to pay more attention to mother of boys during the postpartum home visit, since they might be less likely to use the therapy. Gender-specific support for mothers with male or female infants might be necessary during postpartum period.

8.5.3. Implications for anthropological research

By using a robust experimental design, my study demonstrated a causal relationship between the use of relaxation therapy, reduced maternal stress, and increased weight gain in infants, which provides a better understanding about mother-infant conflict during lactation. Comparatively, as discussed in Chapter 2, except of the MOM study, most previous studies investigating the Trivers' parent-offspring conflict hypothesis from an anthropological perspective were either observational, where the complexity of the inter-relationships (as shown in Figure 8.1) between factors during breastfeeding were difficult to investigate, or used animal models. On the one hand, observational studies could not investigate cause and effect between variables, on the other hand, the findings related to maternal trade-off decisions in animal studies are difficult to compare with studies in humans [373]. In this context, my research has demonstrated that it is feasible to test anthropological theories in humans using a simple experimental approach.

8.6. Directions for Future Research

8.6.1. Suggestions on future study populations and interventions

a) In order to test the general applicability of the relaxation therapy, future RCTs using the relaxation therapy among different populations are suggested, such as in mothers with expected greater tension or different sources of stress (single mothers, mothers of twins); it may also be worth to test the relaxation therapy in mothers from different cultures.

b) Some other relaxation interventions such as progressive muscle relaxation, yoga, and visual imagery techniques that were not compared and tested in my pilot study are suggested to be tested in future studies.

8.6.2. Suggestions on future study design

a) A longer follow-up period is suggested. For example, it would be valuable to look at the effects of relaxation therapy on improving 4-6 months exclusive breastfeeding rate.

b) It may be interesting to follow up the mothers from the present study population to investigate mother-infant factors until (if) they have second children. This could also determine the differences in maternal investment and trade-offs that occur between first and second children.

8.6.3. Suggestions on outcomes and measures

a) Biological sample collection is suggested to be conducted at a standardised time during the day.

b) Stable isotope techniques for the measurement of milk intake and infant body composition are suggested.

c) Analysis of breast milk oligosaccharides is suggested in future research in order to have better understanding about the role of the microbiome in mother-infant signalling

d) Objective assessments for maternal stress and infant behaviour are suggested together with the perceived stress questionnaire. For example, some smart watches have the function of monitoring heart rate during a day and saving data for several days; they could be used to provide objective data on maternal stress and reduce the bias caused by the self-reported questionnaires.

e) Further research should investigate whether there is a significant difference in breastmilk composition between mothers of boys and girls when they receive same duration of the intervention; and whether infant behaviours are significantly different between boys and girls and if that could result in the different usage of relaxation intervention in their mothers.

f) Online-questionnaires which are simple, convenient and easy to use on a smart phone are also suggested, particularly for the measurement of infant behaviour. Since a majority of mothers in my study complained that there were too many questionnaires for them to complete, the design of 3-day infant behaviour is

complicated, these consequently reduced the compliance rate on 3-day infant behaviour questionnaires.

8.7. Conclusions

This thesis has presented my research investigating mother-infant signalling following late preterm and early term delivery. The pilot study evaluated five relaxation techniques among 20 primiparous mothers and identified the relaxation meditation tape was the most effective intervention for mothers who were breastfeeding. The main RCT confirmed the significant effects of relaxation therapy on reducing maternal postpartum stress and increasing infant weight from 1- to 8-weeks. Besides, in the relaxation group, a gender difference was observed regarding the breast milk energy and infant weight gain; mothers of girls produced milk with higher energy at 8-weeks and girls increased more weight than boys. Moreover, non-significant trends suggested that the relaxation therapy might have effects on increasing infant's length gain, and result in higher breast milk energy and fat composition at 8-weeks. Additionally, the microbiome analyses showed that the relaxation intervention led to reduced maternal gut and breast milk microbiome diversity whilst increasing the infant's gut microbiome diversity; it also influenced the change in microbiome from 1 to 8 week. Potential pathways for all these intervention effects were discussed from physiological, psychological, and anthropological perspectives. The study findings have practical implications in terms of supporting breastfeeding mothers following LP and ET delivery. The relaxation meditation tape as a simple and practical tool could easily be used in

future research and in clinical settings to support breastfeeding practice. Findings of this research also contribute to understanding the mechanisms of mother-infant signalling during early life and suggestions for future research are provided.

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Appendix (submitted separately)

1. Pilot study published on Breastfeeding Medicine
2. Ethical approval obtained from University College London and Beijing Children's Hospital
3. Protocol of the randomised controlled trial published on International Breastfeeding Journal
4. Questionnaires used in the research
5. Detailed methods of the microbiome analyses
6. Baseline characteristics of the participants included in the microbiome analyses
7. Rarefaction curve of the microbiome analyses samples