Short-term variations in breastmilk composition: Associations with feeding patterns and gastric emptying in term infants

By

Sadaf Khan (MSc)

This Thesis is presented for the degree of

Doctor of Philosophy (Biochemistry)

THE UNIVERSITY OF WESTERN AUSTRALIA

Faculty of Life and Physical Sciences

School of Chemistry and Biochemistry

35 Stirling Highway, Crawley, Western Australia 6009, Australia

2012
DECLARATION FOR THESSES CONTAINING PUBLISHED WORK AND/OR WORK PREPARED FOR PUBLICATION

The examination of the thesis is an examination of the work of the student. The work must have been substantially conducted by the student during enrolment in the degree.

Where the thesis includes work to which others have contributed, the thesis must include a statement that makes the student’s contribution clear to the examiners. This may be in the form of a description of the precise contribution of the student to the work presented for examination and/or a statement of the percentage of the work that was done by the student.

In addition, in the case of co-authored publications included in the thesis, each author must give their signed permission for the work to be included. If signatures from all the authors cannot be obtained, the statement detailing the student’s contribution to the work must be signed by the coordinating supervisor.

Please sign one of the statements below:

1. This thesis **does not contain** work that I have published, nor work under review for publication.

   Student Signature

2. This thesis contains **only** *sole-authored* work, some of which has been published and/or prepared for publication under sole authorship. The bibliographical details of the work and where it appears in the thesis are outlined below.

   Student Signature

3. This thesis contains published work and/or work prepared for publication, **some of which has been co-authored**. The bibliographical details of the work and where it appears in the thesis are outlined below. The student must attach to this declaration a statement for each publication that clarifies the contribution of the student to the work. This may be in the form of a description of the precise contributions of the student to the published work and/or a statement of percent contribution by the student. This statement must be signed by all authors. **If signatures from all the authors cannot be obtained, the statement detailing the student’s contribution to the published work must be signed by the coordinating supervisor**.

   As detailed in the In statement of candidate contribution (Page V of the Preface), following authors contributed to the published work and/or work prepared for publication listed at the beginning of this thesis:

   Dr Yonja Casadio
   Dr Ching Tat Lai
   Dr Danielle Prime
   Ms Anna Hopworth
   Dr Naomi Tregove
   Dr Donna Geddes
   Professor Peter Hartmann

I, Sadaf Khan, declare that all authors have given the permission for their work to be included in this thesis.

   Student Signature

   Coordinating Supervisor Signature
PREFACE

The work presented in this thesis was supervised by Professor Peter Hartmann, Dr Naomi Trengove and Dr Donna Geddes, School of Chemistry and Biochemistry. My candidature was financially supported by the scholarship from Higher Education Commission, Pakistan and University International Fee Extension Scholarship (March-May 2012). This thesis is presented as a series of scientific papers; two of which are published (2,4) and one is submitted for publication (3). The parts of this body of work have been presented at scientific conferences in both poster and oral formats (4-6).

Research Papers:


Conference Abstracts:

expression session and over a 24-h breastfeeding period – Annual Scientific Meeting of the Nutrition Society of Australia. December 8-11, 2009. Newcastle, Australia.


I am thankful to God for giving me the opportunity and strength to complete my PhD. I am also grateful to many people whose advice and support helped me to complete this thesis. I would like to thank and acknowledge them.

First of all I would like to thank my coordinated supervisor W/Professor Peter Hartmann for his guidance and advice in this research. Thank you very much Peter for your endless support and motivation. I would also like to thank my co-supervisors Dr Naomi Trengove and Dr Donna Geddes for their supervision and support throughout my studies.

I am grateful to Dr Donna Geddes for performing the ultrasound measurements and providing the technical assistance.

I would like to thank Ms Anna Hepworth for providing the help in statistical analysis of my research.

I would also like to thank Dr Ching Tat Lai and Dr Danielle Prime for the useful work related discussions and encouragement.

A very big Thank you to all members of Hartmann Human Lactation Research Group for their support, advice and friendship throughout my PhD.

Thank you to all my UWA friends, especially Hattice Thoma and Tracey Williams, for their love and friendship throughout my stay at UWA.

Finally, I would like to thank my family, my mother and father, my brother and sisters and my grandmother for their love, never ending support and encouragement. I couldn’t have done this without their motivation and support.
STATEMENT OF CANDIDATE CONTRIBUTION

The majority of the work presented in this thesis was undertaken by the author, other individuals also contributed to the publication arising from this thesis. The relative contribution of the manuscript authors is provided below.

Chapter 2: The author performed all the laboratory work, interpretation and analysis of results and drafted the manuscript.
Dr Ylenia Casadio was involved in the study design, and edited the manuscript.
Dr Ching Tat Lai provided the editorial support.
Dr Danielle Prime was involved in the study design, and edited the manuscript.
Ms Anna Hepworth provided statistical and editorial support.
Dr Naomi Trengove provided critical feedback and manuscript editing.
Professor Peter Hartmann supervised this research and provided critical editing of the manuscript.

Chapter 3: The author performed all the laboratory work, interpretation and analysis of results and drafted the manuscript.
Dr Danielle Prime was involved in the study design, provided the samples used in the study, and edited the manuscript.
Ms Anna Hepworth provided the statistical support.
Dr Ching Tat Lai provided the editorial support.
Dr Naomi Trengove provided critical feedback and manuscript editing.
Professor Peter Hartmann supervised this research and provided critical editing of the manuscript.

Chapter 4: The author performed all the laboratory work, interpretation and analysis of results and drafted the manuscript.
Ms Anna Hepworth provided statistical and editorial support.
Dr Danielle Prime and Dr Ching Tat Lai provided the editorial support.
Dr Naomi Trengove provided critical feedback and manuscript editing.
Professor Peter Hartmann supervised this research and provided critical editing of the manuscript.

Chapter 5: The author performed all the laboratory work, interpretation and analysis of results and drafted the manuscript.
Ms Anna Hepworth provided statistical and editorial support
Dr Naomi Trengove provided critical feedback and manuscript editing.
Professor Peter Hartmann was involved in the study design and coordination, and provided critical editing of the manuscript.
Dr Donna Geddes supervised this research, carried out the ultrasonography component of the study, provided the ultrasound images and edited the manuscript.
# TABLE OF CONTENTS

Short-term variations in breastmilk composition: Associations with feeding patterns and gastric emptying in term infants ................................................................. i

PREFACE ........................................................................................................ ii

ACKNOWLEDGMENTS .................................................................................. iv

STATEMENT OF CANDIDATE CONTRIBUTION ............................................ v

TABLE OF CONTENTS .................................................................................. vii

LIST OF FIGURES ........................................................................................ xiii

LIST OF TABLES .......................................................................................... xv

SYMBOLS AND UNITS ................................................................................ xvi

ABSTRACT ...................................................................................................... xvii

CHAPTER 1  Literature Review ..................................................................... 1

1.1 Introduction ............................................................................................ 1

1.2 Breast Anatomy ..................................................................................... 4

1.3 Milk Synthesis ....................................................................................... 5

1.4 Control of Milk production .................................................................... 7

1.5 Milk Composition ................................................................................ 9

1.5.1 Milk Fat ............................................................................................ 10

1.5.2 Carbohydrates .................................................................................. 12

1.5.3 Proteins ........................................................................................... 13

1.5.3.1 Whey .......................................................................................... 14

1.5.3.2 Caseins ..................................................................................... 16
1.5.3.3 Milk fat globule membrane proteins .................................................. 18

1.5.4 Energy .......................................................................................................... 19

1.6 Factors affecting milk composition ................................................................. 20

1.7 Infant feeding patterns .................................................................................... 24

1.7.1 Self-regulation of breastmilk intake ............................................................. 24

1.8 Gastric emptying (GE) ...................................................................................... 25

1.8.1 Physiology and Control of GE ..................................................................... 26

1.8.2 Patterns and rates of GE in infants .............................................................. 28

1.9 Factors affecting GE ....................................................................................... 30

1.9.1 Milk Composition ....................................................................................... 31

1.9.2 Casein Curding ............................................................................................ 32

1.9.3 Feed Volume ............................................................................................... 33

1.10 Methods for assessing GE ............................................................................. 34

1.11 Research Aims ............................................................................................... 37

CHAPTER 2 Investigation of short-term variations in casein and whey proteins in
breastmilk of term mothers ..................................................................................... 38

2.1 ABSTRACT ....................................................................................................... 38

2.2 INTRODUCTION ............................................................................................. 39

2.3 MATERIALS AND METHODS ....................................................................... 41

2.3.1 Subjects ....................................................................................................... 41

2.3.2 Sampling ..................................................................................................... 42

2.3.3 24h Milk production measurements ............................................................ 42

2.3.4 Isolation of casein and whey ..................................................................... 43

2.3.5 Protein assay .............................................................................................. 44
2.3.6 Statistical Analysis........................................................................................................................................44
2.4 RESULTS ...........................................................................................................................................................46
  2.4.1 Participants ................................................................................................................................................46
  2.4.2 Separation of casein and whey ....................................................................................................................46
  2.4.3 Protein concentration in fore and hind milk ...............................................................................................47
  2.4.4 Circadian and Inter-individual variation in protein concentration .........................................................49
  2.4.5 Protein concentration between breasts ....................................................................................................51
2.5 DISCUSSION ....................................................................................................................................................52

CHAPTER 3 Investigation of short-term variations in term breastmilk composition during repeated breast expression sessions .................................................................................................................................57
  3.1 ABSTRACT .....................................................................................................................................................57
  3.2 INTRODUCTION .............................................................................................................................................58
  3.3 MATERIAL AND METHODS .........................................................................................................................60
    3.3.1 Subjects ...................................................................................................................................................60
    3.3.2 Sampling ................................................................................................................................................60
    3.3.3 Mid-Infrared Analysis .............................................................................................................................61
    3.3.4 24h Milk Production Measurements ....................................................................................................61
    3.3.4 Creamatocrit Analysis ............................................................................................................................62
    3.3.5 Statistical Analysis ..................................................................................................................................62
  3.4 RESULTS ........................................................................................................................................................64
    3.4.1 Participants ............................................................................................................................................64
    3.4.2 Milk composition over time and between breasts ..................................................................................64
    3.4.3 Milk composition and expression characteristics ..................................................................................68
3.4.4 Comparison of fat content from single expression session to the 24h fat value
....................................................................................................................................... 69

3.5 DISCUSSION .................................................................................................................... 70

CHAPTER 4 Variation in fat, lactose and protein composition in breastmilk over 24h:
Associations with infant feeding patterns ............................................................................ 75

4.1 ABSTRACT ......................................................................................................................... 75

4.2 INTRODUCTION ................................................................................................................. 76

4.3 MATERIAL AND METHODS ............................................................................................ 78

4.3.1 Subjects ......................................................................................................................... 78

4.3.2 Milk Sampling ................................................................................................................ 79

4.3.3 Biochemical analysis ..................................................................................................... 79

4.3.3.1 Milk fat ...................................................................................................................... 79

4.3.3.2 Milk lactose ............................................................................................................... 79

4.3.3.3 Milk protein ................................................................................................................. 79

4.3.3.4 Milk energy ................................................................................................................ 80

4.3.4 Measurement of 24h infant milk intake .................................................................... 80

4.3.5 Determination of 24h nutrient and energy intake ..................................................... 81

4.3.6 Statistical analysis ........................................................................................................ 82

4.4 RESULTS .......................................................................................................................... 83

4.4.1 Breastfeeding characteristics ....................................................................................... 83

4.4.2 Milk fat ......................................................................................................................... 84

4.4.3 Milk lactose .................................................................................................................. 85

4.4.4 Milk protein .................................................................................................................. 87

4.4.5 Milk energy ................................................................................................................... 87
CHAPTER 5  Ultrasonic assessment of gastric volume and emptying in term breastfed infants .................................................................................................................................................................................. 96

5.1 ABSTRACT ................................................................................................................................................................................................................................................ 96

5.2 INTRODUCTION ....................................................................................................................................................................................................................... 97

5.3 MATERIAL AND METHODS .............................................................................................................................................................................................. 100

5.3.1 Participants ......................................................................................................................................................................................................................... 100

5.3.2 Sampling ............................................................................................................................................................................................................................. 101

5.3.3 Biochemical Analysis ............................................................................................................................................................................................... 101

5.3.3.1 Milk Fat .................................................................................................................................................................................................................. 101

5.3.3.2 Milk Lactose ........................................................................................................................................................................................................... 102

5.3.3.3 Milk Protein ........................................................................................................................................................................................................ 102

5.3.3.4 Milk Energy ..................................................................................................................................................................................................... 103

5.3.4 Ultrasound Examination .......................................................................................................................................................................................... 103

5.3.4.1 Gastric volume measurement ........................................................................................................................................................................... 104

5.3.4.2 Gastric emptying assessment .............................................................................................................................................................................. 104

5.3.4.3 Ultrasound imaging of gastric content ......................................................................................................................................................... 105

5.3.5 Determination of Milk intake ................................................................................................................................................................................... 107

5.3.6 Statistical Analysis ............................................................................................................................................................................................... 107

5.3.6.1 Reliability of ultrasound measurements .................................................................................................................................................... 108

5.3.6.2 Validation of gastric volume measurements ................................................................................................................................................ 109

5.3.6.3 Comparison of ultrasound image based quantification and feeding characteristics .................................................................................................................. 109
5.3.6.4 Relationship between gastric emptying and milk intake and composition

................................................................. 110

5.4 RESULTS ........................................................................................................ 111

5.4.1 Participant characteristics.............................................................................. 111

5.4.2 Reliability of ultrasound measurements......................................................... 111

5.4.3 Gastric volume and Milk intake ...................................................................... 113

5.4.4 Post-feed ultrasound imaging of gastric contents ........................................... 114

5.4.5 Ultrasound image based quantification and feeding characteristics............... 115

5.4.6 Gastric Emptying (GE) .................................................................................. 118

5.5 DISCUSSION ..................................................................................................... 120

CHAPTER 6 General Discussion ........................................................................... 128

REFERENCES ........................................................................................................ 137
LIST OF FIGURES

Figure 1.1: Motor events associated with normal gastric emptying. .......................... 28

Figure 1.2: Patterns of emptying of solid and liquid meal. ........................................ 29

Figure 2.1: Gel electrophoresis of separated skim milk, whey and casein fractions. .... 47

Figure 2.2: Distribution of protein concentration of skim, whey and casein fractions in
fore and hind milk collected during breast expression................................. 48

Figure 2.3: Distribution of protein content of skim milk, whey and casein fraction of
milk samples taken during breastfeeding in the morning, day, evening and night.
.......................................................................................................................... 51

Figure 2.4: Comparison of protein concentration between breasts in skim, whey and
.casein fractions across four time points of the day............................................. 52

Figure 3.1: Distribution of fat, protein, lactose, total solid and energy content of
breastmilk across three weeks. .............................................................. 66

Figure 3.2: Difference in fat, protein and lactose concentration between breasts in
individual mothers. ......................................................................................... 67

Figure 3.3: Pattern of variation in fat, total solids and energy in each of the 23 mothers.
.......................................................................................................................... 68

Figure 3.4: Difference between mean fat content from a single expression session (one
off sample of 24h) and 24h fat content for left and right breast in individual
mothers.................................................................................................................. 70

Figure 4.1: Patterns of breastfeeds over 24h in individual mother-infant pairs............ 84
Figure 4.2: Distribution of fat and lactose concentration in pre- and post-feed milk collected during breastfeeding over 24h. ................................................................. 86

Figure 5.1: Ultrasound images of the infants’ stomach immediately after the feed... 106

Figure 5.2: Comparison of observed post-feed curd density in the stomach with spleen. .......................................................................................................................... 107

Figure 5.3: Bland-Altman plots showing the difference between the first and second replicates of the rater for each of the ultrasound measurement. ..................... 112

Figure 5.4: Milk intake of the infant in relation to the stomach volume measured by ultrasound........................................................................................................... 114

Figure 5.5: Association between ultrasound image based classification of stomach echogenicity observed at the end of the feed to the macronutrient content of the milk samples. ............................................................................................................. 116

Figure 5.6: Association between ultrasound image based classification of post-feed Curd density (A) and Curd volume (B) to the macronutrient content of the samples. .................................................................................................................. 117

Figure 5.7: Box plot analysis of the association between stomach echogenicity, curd density and curd volume grading to the milk volume taken during the feed...... 118

Figure 5.8: Pattern of gastric emptying in individual infants. ................................................. 120
LIST OF TABLES

Table 3.1: Breastmilk macronutrient concentration across weekly intervals.............. 65
Table 3.2: Breastmilk macronutrient concentration at follow up periods............... 65
Table 4.1: Milk composition and breastfeeding pattern over a 24h period .............. 89
Table 4.2: Macronutrient concentration and 24h infant nutrient intake from left and
right breasts ............................................................................................................. 90
Table 5.1: Mean, SD and limits of agreement of the difference between replicates
(rep2-rep1) and ICC agreement and consistency scores by rater....................... 113
## SYMBOLS AND UNITS

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>nm</td>
<td>Nanometer</td>
</tr>
<tr>
<td>mm</td>
<td>Millimeter</td>
</tr>
<tr>
<td>µl</td>
<td>Microlitre</td>
</tr>
<tr>
<td>ml</td>
<td>Millilitre</td>
</tr>
<tr>
<td>l</td>
<td>Litre</td>
</tr>
<tr>
<td>Kcal</td>
<td>Kilocalorie</td>
</tr>
<tr>
<td>kJ</td>
<td>KiloJoule</td>
</tr>
<tr>
<td>sec</td>
<td>Second</td>
</tr>
<tr>
<td>min</td>
<td>Minute</td>
</tr>
<tr>
<td>h</td>
<td>Hour</td>
</tr>
<tr>
<td>g</td>
<td>Grams</td>
</tr>
<tr>
<td>g/l</td>
<td>Grams per litre</td>
</tr>
<tr>
<td>g</td>
<td>Force of Gravity</td>
</tr>
<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>mM</td>
<td>MilliMolar</td>
</tr>
<tr>
<td>ºC</td>
<td>Degree Celsius</td>
</tr>
<tr>
<td>24h</td>
<td>24 hour period</td>
</tr>
<tr>
<td>kD</td>
<td>kiloDalton</td>
</tr>
<tr>
<td>α</td>
<td>Alpha</td>
</tr>
<tr>
<td>β</td>
<td>Beta</td>
</tr>
<tr>
<td>κ</td>
<td>Kappa</td>
</tr>
<tr>
<td>CV</td>
<td>Coefficient of variation</td>
</tr>
</tbody>
</table>
ABSTRACT

Breastmilk is the ideal form of nutrition for the normal growth and development of newborn babies. Breastfed infants vary greatly in their feeding frequency and in the volume of milk taken at each breastfeed throughout the day. This variation suggests that a self-regulatory mechanism determines milk intake from feed to feed in breastfed infants. Milk consumption by breastfed infants can be affected by either maternal (e.g. short-term variation in milk composition) or infant factors (e.g. gastric emptying rates) or a combination of both.

Little research has been undertaken on gastric emptying of breastfed infants, it is likely that variation in the composition and volume of breastmilk alters the rate of gastric emptying. Mostly studies have used formula milk to investigate the effect of nutrient content on gastric emptying in infants and report that factors such as high levels of fat, carbohydrate and protein, together with the high energy density of formula resulted in delayed gastric emptying. Therefore, it is important to investigate whether the same relationships occur with breastmilk, as this would help in understanding the regulation of energy intake in breastfed infants.

This project aims to characterize the short-term variations in milk composition and to establish the relationship between maternal (breastmilk composition, particularly whey and casein proteins) and infant factors (breast feeding patterns and gastric emptying).

Breastmilk samples were collected from healthy term infant mothers from each breast at each breastfeed over a 24h period (n=15) and on three occasions within three weeks (n=23) from the simultaneous breast expression. 24h milk intake of the infants was also determined. In addition, mother-infant dyads (n=27) were studied during one
feed (n=18), then at 10-15min intervals (n=9) to monitor stomach volume using ultrasound, and the post-feed echogenicity of stomach contents was assessed and graded.

Casein and whey proteins provide both nutritional and physiological benefits to infants. In addition, these proteins are considered to be an important factor influencing gastric emptying in formula fed infants. No circadian variations were observed in whey (7.6±1.5g/l) and casein (3.4±0.97g/l) concentration over the 24h breastfeeding period. No weekly (over a three week period) variation was found in breastmilk fat content, protein and lactose concentrations, in relation to the expression volume. This establishes that when sampling for nutritional studies it is not necessary to account for these short-term variations.

The relationship between breastmilk macronutrient concentration, individual milk proteins and patterns of milk intake in breastfeeding infants over a 24h breastfeeding period was examined. Breastfeeding patterns and milk composition varied greatly between individuals with CVs of 27.0%, 15.5%, 19.8% and 28.4% for fat, total protein, whey and casein, respectively. The mean 24h total protein, whey and casein intake were inversely (p<0.01) whereas lactose concentration was positively (p=0.03) related to the number of breast feeds per day. No relationship was seen either between fat or energy content and feeding patterns. The association between milk protein intake and the breastfeeding frequency suggests that the protein intake may play a role in infant appetite control.

Ultrasound was successfully validated by test weighing technique, which is used for determination of milk intake (p<0.001, R²=0.90) as a sensitive and accurate method to monitor gastric emptying in term infants and was also used to visualize the gastric
contents. Different patterns and echogenicity of stomach contents were displayed immediately after the feed and were related to the casein concentration (p=0.02) of the milk samples. The emptying patterns observed were curvilinear. No significant relation was determined between breastfeed macronutrient content and gastric emptying. However, gastric emptying was inversely related to the feed volume (p=0.04). In conclusion, the volume and colligative property of breastfeed appears to play a role in the control of gastric emptying in breastfed term infants and is likely to affect the satiety and consequently the regulation of food intake in developing infant.
CHAPTER 1  Literature Review

1.1 Introduction

Breastmilk is unequivocally the optimal form of nutrition for healthy term infants all over the world. Breastmilk contains essential nutrients, bioactive and immunological components that provide species-specific nutrition, protection against infectious agents and psychological benefits to ensure normal infant growth and development (Lonnerdal, 2000). Moreover, it may be an advantage that breastfed infants not only self-regulate their energy intake but also do so at a lower level than that consumed by formula-fed infants (Dewey, 1998), which may provide protection against obesity risks later in life. Therefore, the current recommendations to mothers are that infants should be breastfed “on demand” (according to their appetite) exclusively for the first six month of life, and after that with the inclusion of complementary foods for at least two years (World Health Organization, 2001; NH&MRC, 2003).

Breastmilk composition is not uniform and varies both within and between mothers according to the growing needs of the infant. There are number of different factors including the short-term factors (such as between feed variations, circadian rhythm, weekly intervals etc) that influences the milk composition (fat, protein, lactose and energy content) and volume. It is possible that either these changes or differences in nutrient content could influence the infant milk intake; therefore, it is important to characterize these short-term variations both within and between mothers.

Longitudinal changes have been reported not only in total protein concentration but also in the proportion of two main classes of breastmilk whey and casein. The total
protein concentration declines during lactation where as casein concentration increases with a simultaneous decrease in the concentration of total whey proteins (Kunz and Lonnerdal, 1992). As a result the ratio of whey to casein is not fixed in human milk and varies throughout lactation from 90:10 in early lactation to 60:40 in mature milk (Kunz and Lonnerdal, 1992). In addition, temporal variations have been reported in the immune components and complement system proteins of the breastmilk (Franca et al., 2010). These proteins were found to be higher during the daytime, which provides extra protection to the infant since daytime provides more chances for an infant to be exposed to infectious agents (Franca et al., 2010). However, to our knowledge there is no study that has investigated the circadian variation in whey and casein proteins, which provide both immunological and nutritional benefits. This information will help in understanding the role of milk proteins in the short-term control of milk intake.

In exclusively breastfed infants with breastmilk as their only source of nutrition, behaviour control of food intake is accomplished through alterations in dietary patterns such as frequency of feeding, interval between feeds and size of the feed (Matheny et al., 1990). Marked variations have been reported in feeding patterns and daily milk intake within and between mother infant pairs throughout the day (Butte et al., 1984b; Kent et al., 2006; Dewey and Lonnerdal, 1983), with the daily milk intake range from 500-1300ml per day during the first 6 months of life (Kent et al., 2006). It has been well established that breastfed infants self regulate their milk intake and feed according to their appetite as infants do not stop feeding because the breast is empty and on average consume only 67% of the available milk in the breast (Kent et al., 2006). However, data regarding the association between changes in breastmilk
composition and caloric content and infant’s level of demand is limited. Therefore, there is a need to investigate how breastmilk composition particularly protein composition (casein and whey) influences the infant feeding patterns since protein composition has been reported as an important factor contributing to stomach emptying (Khoshoo and Brown, 2002). The two main classes of milk proteins casein and whey behave differently during digestion, and casein precipitates and form curd under the acidic gastric condition, which slows it’s emptying and could influence the feeding behaviour of infants (Khoshoo and Brown, 2002; Anderson and Moore, 2004). Gastric emptying in synergy with other factors such as hormonal factors is considered to play an important role in regulation of appetite and has also been implicated in the control of feeding behaviour in human adults and animals (Lorenz, 1985; Hellstrom et al., 2006). The key factors that are known to influence gastric emptying are the volume and the composition of the feed (Siegel et al., 1985; Hunt and Stubbs, 1975). However, most of our knowledge about the effect of milk composition on gastric emptying is based on formula milk (derived from cow’s milk) and these studies are mostly carried out in preterm infants. There are several compositional differences between breast and formula milk particularly the different protein composition and the whey to casein ratio of formula milk, which is manipulated to mimic the ratio assumed to be present in breastmilk. These differences may result in delayed emptying in formula-fed infants as compared to breastfed infants, which is considered a reason for the low number of feeds in the former (Cavell, 1981). In addition, preterm infants are different from term infants in their nutritional requirements and also have less developed body systems. Previous studies have also not commented about the casein concentration and gastric curding between infants, a factor that digests more slowly than of the other milk
components. Therefore, there is a need to investigate the relationship between breastmilk composition (particularly the effect of curding) and gastric emptying in term infants, which will help in understanding the regulation of food intake in infants. Furthermore, a suitable non-invasive technique that does not involve radiation hazard is required to monitor the gastric emptying in infants. Ultrasound has previously been used to monitor gastric emptying in pre-term infants and children (Newell et al., 1993; Gomes et al., 2003) but has not been validated in term infants. Therefore, validation of the technique is required to evaluate gastric emptying in relation to breastmilk composition.

1.2 Breast Anatomy

The breast is composed of glandular (secretory) and adipose (fatty) tissues, and is supported by connective fibres called Cooper’s ligaments that form the structural framework of the breast. The glandular tissue is composed of lobes, which are comprised of lobules containing groups of alveoli (Geddes, 2007b). During lactation, the alveoli are lined with secretory mammary epithelial cells known as lactocytes that synthesise and secrete milk components into the lumen of alveoli. The alveoli are surrounded by myoepithelial cells, which in response to oxytocin contract and eject the milk from the alveolar lumen in to small ducts leading from alveoli (Berry C A et al., 2007). These small ducts join to form larger ducts draining the lobules, and larger ducts finally merge into one milk duct for each lobe. There are on average 9 milk ducts (range 4-18) that terminate at the nipple surface in each lactating breast (Geddes, 2007b).
The adipose tissue is variably distributed in the breast and present beneath the skin (subcutaneous), between the glandular tissue (intra-glandular) and behind the breast (retromammary fat) (Ramsay et al., 2005; Geddes, 2007b). The lactating breast is predominantly composed of glandular tissue, and is present in the ratio of approximately 2:1 to adipose tissue (Ramsay et al., 2005). Despite this predominance, no relationship has been found between the amount of glandular tissue with the storage capacity and milk production of the breast (Ramsay et al., 2005), which is consistent with milk production being regulated by the infant’s appetite (Dewey and Lonnerdal, 1986).

The major blood supply of the breast is from internal mammary and lateral thoracic arteries (Cunningham, 1977), and mammary blood flow has been shown to change during breastfeeding (Janbu et al., 1985). There is limited distribution of nerve fibres in the breast glandular tissue when compared to the nipple and areola where sensory innervation is extensive and consist of both autonomic and sensory nerves (Montagna and Macpherson, 1974). Apart from some sensory nerves fibres found near the major ducts of the breast, the nerve supply to the inner gland is sparse with the majority of the mammary nerves following blood vessels (Montagna and Macpherson, 1974; Lawrence and Lawrence, 2010). In addition, there is no evidence of motor innervations of lactocytes and glandular tissue, which supports that milk production, is independent of neural stimulation (Geddes, 2007a; Lawrence and Lawrence, 2010).

### 1.3 Milk Synthesis

The breast undergoes massive anatomical changes due to the action of certain hormones and molecular factors during pregnancy, to attain its full functional capacity
to synthesize milk constituents. The process of initiation of lactation (lactogenesis) occurs in two phases: secretory differentiation (lactogenesis I) and secretory activation (lactogenesis II)(Neville and Robert, 1995a; Czank et al., 2007a; Pang and Hartmann, 2007).

Secretory differentiation begins in the second half of pregnancy and involves the differentiation of alveolar epithelial cells into lactocytes. The differentiated lactocytes has the potential to synthesise many specific milk components including, lactose, casein and $\alpha$-lactalbumin. During this phase, a thick, yellowish and immature form of milk is produced in small quantity ($\approx$30mL each day); this milk is known as colostrum. It contains high concentrations of protein, sodium and chloride and low amounts of lactose, citrate and potassium. Colostrum is important to the newborn baby as it contains higher amounts of immunoprotective proteins like secretory IgA (sIgA) and lactoferrin, which provide protection against pathogenic organisms. During this phase, the tight junctions between the lactocytes are not closed, allowing lactose, one of the secreted components, to enter into the blood stream through the paracellular pathway and then excreted into urine (Neville et al., 1983). Therefore, the urinary lactose levels can be used as an indicator of secretory differentiation (Cox et al., 1999).

The ‘lactogenic hormone complex’ of the reproductive hormones, estrogen, progesterone, prolactin and some metabolic hormones play the major roles during this process.

Secretory activation is associated with the initiation of copious milk secretion and occurs after parturition. This phase requires the hormones estrogen, prolactin, insulin and glucocorticoids, and is triggered by the withdrawal of circulating progesterone at parturition in women. Mothers sense lactogenesis II as a sudden feeling of breast
fullness as the milk comes in at about 30-40 hours postpartum.

During secretory activation, the tight junctions between the lactocytes are closed, therefore the synthesized milk components are retained in alveolar lumen, which result in an increase in milk secretion. Milk production in women increases between 395-868ml in the first few days of lactation and by 1 month it averages 750 to 800ml (Kent, 2007). Then over the first six months of lactation, milk production for each baby is relatively constant, where a daily milk production of 487 to 1356mL is considered within the normal range (Kent et al., 2006).

Lactogenesis II not only results in an increase in milk production, but the composition of milk also changes during this phase. At about 1-2 days after the onset of lactogenesis II the milk becomes thinner as result of fully productive breast and the concentration of protective proteins, sodium and chloride decreases while the concentration of fat, lactose, citrate and potassium increases. Therefore, these changes in milk components and the milk production have been used as biochemical markers of lactogenesis II (Arthur et al., 1989; Cregan et al., 2002). However, regardless of the production of mature milk and stabilization of milk production, large variations have been reported in composition and production of milk both within and between mothers (Mitoulas et al., 2000).

1.4 Control of Milk production

Once lactation is established regular emptying of the breast is required to sustain this copious milk supply (Neville et al., 2002), since ineffective drainage of the gland will down-regulate milk production, and trigger apoptosis and involution of the gland (Peaker and Wilde, 1996). The maintenance of milk production is regulated by the
removal of milk either by suckling or expression during established lactation. Suckling maintains milk production by the release of endocrine hormones, most important of which are prolactin and oxytocin, as well as by stimulating autocrine (local) control (Hartmann PE, 1991).

Prolactin released from the anterior pituitary in response to a breastfeed or expression stimulates milk synthesis, and reaches a peak in concentration at about 45 minutes following stimulus (Noel et al., 1974). The binding of prolactin to its receptors on the surface of lactocytes activates the signal transduction pathway, which regulates the expression of genes involved in the synthesis of milk proteins, such as casein and α-lactalbumin. In addition, this hormone also regulates lipogenesis in the mammary gland (Martyn and Falconer, 1985). Prolactin plays an important role in the establishment and maintenance of lactation (Ostrom, 1990), as its absence stop milk production. Prolactin levels are high in early lactation, however during established lactation only lower basal levels are required to maintain production (Cowie et al., 1980). Therefore, prolactin levels decline over the first six months of lactation (Cox et al., 1996), while milk production remain relatively constant during this period. In this regard, it has been concluded that prolactin blood levels do not directly control the amount of milk produced (Cox et al., 1996).

While prolactin is responsible for milk synthesis, oxytocin released from the posterior pituitary in response to suckling, facilitates milk removal. Oxytocin causes the contraction of the myoepithelial cells surrounding the alveoli thus forcing the expulsion of milk from the alveoli into the milk ducts. This is termed the milk-ejection reflex, without which little or no milk can be removed from the breast (Prime et al., 2007). The number of milk ejections influences the amount of milk taken by the baby
and is independent of the length of the breastfeed (Ramsay et al., 2004). However, it has also been found that the baby may terminate more than one third of the breastfeeds during a milk ejection, suggesting that infant control of milk intake is not limited by number of milk ejections (Ramsay et al., 2004). Therefore, the control of milk synthesis during established lactation appears to be determined by the local, autocrine system that responds to infant’s demand for milk (or appetite) (Daly et al., 1993b). The rate of milk production within each breast responds to the degree to which the breast has been emptied i.e. the greater the degree of emptying or milk removal; the greater will be the rate of milk synthesis. This could possibly occur by removal of a chemical inhibitor in the milk, called feed back inhibitor of lactation (FIL) (Daly et al., 1993b; Prentice et al., 1989).

FIL a 7.5kD acidic whey protein produced by lactocytes has been indentified in the milk of goats, women and cows. It has been shown that FIL acutely regulates milk synthesis and secretion in a dose dependent manner (Wilde et al., 1998; Wilde et al., 1995). This concentration dependent nature of the inhibition suggests that the concentration of FIL increases when milk is accumulated within the mammary gland that slows milk production, and is decreased upon milk removal, which speeds up the production (Wilde et al., 1995). Despite the good evidence of local control effects of FIL, the mechanism of action of FIL is still unclear.

1.5 Milk Composition

Human milk is a complex mixture of proteins, fats, carbohydrates, trace elements, minerals, vitamins, growth factors, bioactive and cellular components that in addition to providing energy and protection are also involved in the growth and development
of the breastfed infants. Milk components are compartmentalized into emulsified globules (e.g. fat), micelles (colloidal dispersion of casein and minerals) and into true solution (remainder of proteins, minerals and carbohydrates) (Czank et al., 2007c), which control their flow into the infant gut when milk has been consumed (Jensen et al., 1991).

Furthermore, milk composition is not uniform and may change according to the growing needs of the infant.

1.5.1 Milk Fat

Milk fat is important for the maturation and development of the infant, and provides more than half of the infant’s energy requirements. The fat fraction of human milk is composed of several different classes of lipids. More than 98% of the total milk fat is in the form of triacylglycerols, and the reminder consists of di- and monoacylglycerols, nonesterified fatty acids (NEFA), phospholipids, cholesterol and cholesterol esters (Czank et al., 2007c).

The triacylglycerols are hydrolyzed to free fatty acids and glycerol by the action of lipase, which is present both in infant gut and in human milk. The fatty acid in human milk is about 85% of triacylglycerides and consists of over 200 different types including saturated, mono and poly unsaturated, branched and cyclic fatty acids, most of them present in amounts of less than 1% (Jensen, 1999).

The fatty acids required for formation of triacylglycerols are either synthesized within the gland (de novo synthesis) or taken up from the blood lipids, which can be derived from diet, mobilization of adipose tissues or synthesized by the liver. The contribution of lactocytes to fatty acid synthesis is very low and accounts for only 20% of the total fatty acids in mature milk. The mammary gland lactocytes mainly synthesize medium
chain fatty acids (MCFA) (C12-14) using glucose as the dominant substrate (Neville et al., 1983). As lactocytes contain unique thioesterase (thioesterase II), which terminate fatty acids synthesis at the 14th carbon, all other fatty acids such as long chain polyunsaturated fatty acids (LCPUFA), are derived from the blood and are mainly affected by maternal diet (Jensen et al., 1995).

Following synthesis the triacylglycerols are aggregated to form the fat globules. These milk fat globules (MFG) of around 4µm are mainly composed of a triacylglycerol core, which is coated with a membrane known as milk fat globule membrane (MFGM)(Jensen, 1999). MFGM originates from the plasma membrane of lactocytes during secretion of MFG and consist of phospholipids, proteins, mucopolysaccharides, cholesterol, and enzymes. It has been suggested that the size of milk fat globules could have an influence on fat metabolism and gastric emptying rate. For example, in human adults and small animal models, when compared to small-homogenized fat globules, ingestion of large native globules result in faster gastric emptying (GE)(Michalski et al., 2005a). However, in infants, the effect is not clear with studies either showing slower GE (Cavell, 1981) or no significant difference (Armand et al., 1996) when infant formula is ingested whose fat globule size on average ranges from 0.3-1.1microns(Michalski et al., 2005b).

It has been suggested that this discrepancy could be due to the differences in the interface composition of fat droplets. The fat droplets coated with casein trapped within gastric coagulum, could delay their gastric emptying. In contrast, phospholipid coated droplets would not interact with caseins and could drain more easily (Michalski et al., 2002).
1.5.2 Carbohydrates

The principal carbohydrate in human milk is the disaccharide lactose, which is one of the main nutritive components of milk, contributing 40% of the energy delivered to the infant. Human milk also contains monosaccharides (mainly glucose and galactose) and more than 130 different oligosaccharides in lower concentrations (Czank et al., 2007b).

Lactose is comprised of glucose and galactose linked together by 1,4 β-glycosidic bond, and is synthesized in the golgi secretory vesicle system of lactocytes from the transported glucose and UDP-galactose (Neville et al., 1983). An enzyme complex called lactose synthase consisting of a membrane bound enzyme galactosyl transferase, and a regulatory protein α-lactalbumin catalyse this reaction. Once synthesized lactose is trapped in the luminal spaces of the golgi complex and packaged into golgi secretory vesicles. These vesicles migrate towards the apical membrane of lactocytes where they eject their contents by exocytosis, which results in secretion of water, water-soluble components, casein micelles, and some monovalent ions (Mather and Keenan, 1998).

In addition to providing energy, lactose is the major osmotically active component of human milk. Since the golgi membrane is impermeable to lactose, its synthesis results in drawing water into the milk as it is secreted to maintain osmotic equilibrium (Neville et al., 1983). Therefore, the rate of lactose synthesis exerts a major control over milk production (Arthur et al., 1989).

Lactose is hydrolysed into its components by the bush border enzyme lactase in the small intestine and then delivered to the liver.
Oligosaccharides are complex carbohydrates of three to ten monosaccharide subunits and are comprised of glucose, galactose, N-acetylglucosamine, fucose, sialic acid and lactose, which is usually present at reducing end (Coppa et al., 1993; McVeagh and Miller, 1997). Their total concentration in mature human milk is 12.9 g/l, which is comparable to that of total protein (Coppa et al., 1993). They are thought to have immunological activity in infants, such as preventing the binding of pathogens to epithelial cells and promoting the growth of beneficial bacteria, *Bifidobacterium bifidum* in the intestine (Bode, 2006).

### 1.5.3 Proteins

Human milk proteins provide nutritional, developmental and immunological benefits to infants, and can be divided into three compartments: caseins, whey proteins and milk fat globular membrane proteins (Lonnerdal, 2003). Unlike bovine milk, human milk contains less total protein but of a higher biological value, of which the whey fraction constitutes the major part of protein (~60-70%), whereas casein is a smaller fraction (~30-40%) (Lopez Alvarez, 2007; Lonnerdal, 2003). In addition, milk fat globule membrane proteins and proteins present in other cell fractions contain a minor proportion of total protein content of milk (~ 4%) (Czank et al., 2007d). Difficulties have been reported in the complete separation of the casein from whey proteins in human milk. In the case of bovine milk, adjustment of the pH of the milk to the isoelectric point of casein (pH4.6) followed by ultracentrifugation leads to complete separation of two fractions (casein and whey). However, in the case of human milk this approach of acid precipitation of casein leads to co-precipitation of whey proteins. Kunz and Lonnerdal have modified this precipitation process for human milk by adjusting the pH of milk to 4.3 and the addition of 5–60 mM calcium chloride
followed by ultracentrifugation at 189 000g for 1 h at 4°C (Kunz and Lonnerdal, 1992; Kunz and Lonnerdal, 1990b). This procedure appears to show the aggregation of the casein subunits. However, minute contaminations of the two fractions are still reported (Sanchez-Hidalgo et al., 1998).

The majority of milk proteins including caseins and several whey proteins (lactoferrin, α-lactalbumin, whey acidic proteins) are synthesized within lactocytes by the rough endoplasmic reticulum from free amino acids (Burgoyne and Duncan, 1998). After synthesis they are packaged into golgi secretory vesicles and secreted into milk by exocytosis (Neville et al., 1983). Other milk proteins (serum albumins, immunoglobulins and some hormones) are taken up from maternal circulation by endocytosis at the basolateral membrane. These proteins are then transported through the lactocytes and may be released directly into the alveolar lumen or secreted with other milk proteins by exocytosis (Mather and Keenan, 1983).

1.5.3.1 Whey

Whey contains the proteins, which are soluble in the liquid fraction (i.e. whey) that remains after the isoelectric precipitation of caseins with acid in the presence of calcium (Lopez Alvarez, 2007). The whey proteins are very diverse and proteomic studies have identified up to 152 proteins in this fraction (Liao et al., 2011; Palmer et al., 2006). These proteins have been classified into eight functional groups including proteins involved in cell communication, growth/maintenance, nucleic acid regulation, immune response, transport, protein metabolism, energy production pathways and multifunctional proteins (Liao et al., 2011).

The principal whey proteins include lactoferrin, lysozyme, α-lactalbumin, immunoglobulins, hormones, enzymes and binding proteins.
The whey proteins lactoferrin along with lysozyme and sIgA contribute to bacteriostatic and bactericidal system of human milk (Hamosh, 1998).

Lactoferrin is an iron binding glycoprotein with a molecular mass of 78-80kD, consisting of two Fe$^{3+}$ binding lobes connected by an α-helix. Lactoferrin facilitates iron absorption via a lactoferrin receptor present on enterocytes (Suzuki et al., 2001), to provide iron and other trace elements to infants. Due to its high affinity for iron, lactoferrin withholds iron from iron requiring bacteria therefore exerting bacteriostatic activity (Lonnerdal, 2003). In addition, several other functions of lactoferrin have also been demonstrated including cell growth regulation, anti-inflammatory activity, antitumor activity and certain enzyme activities (Hamosh, 1998; Lopez Alvarez, 2007).

Lysozyme a 15kD glycoprotein catalyses the hydrolysis of β-1, 4 linkages between N-acetylmuramic acid and N-acetyl glucosamine in bacterial cell walls, and thus lyses mostly gram-positive and a few gram negative bacteria (McKenzie, 1996).

Human milk contains several immunologic factors that protect infants from infections. sIgA is the major immunoglobulin in human milk, and is present in concentrations of 1-2g/l in early lactation and 0.5-1g/l in late lactation (Goldman, 1993). Several other classes of serum immunoglobulins like IgM and IgG is also present in milk. sIgA is secreted as a dimmer linked together with secretory chains, and because of this molecular arrangement it is resistant to intestinal proteolysis (Goldman, 1993). The mother’s acquired immunity can be transferred to the breastfed infant in the form of sIgA, via the enteromammary pathway, which assists in protecting the infant’s immature immune system (Lonnerdal, 2003).

Apart from immunologic components, the whey portion also consists of nutritive proteins including α-lactalbumin and serum albumin.
α-Lactalbumin is a 14kD protein, constitutes 10-20% of total protein in human milk (Lonnerdal and Atkinson, 1995). Structurally, it consists of single unglycosylated and unphosphorylated polypeptide chain of 123 amino acids and binds Ca$^{2+}$ and Zn$^{2+}$. However, α-lactalbumin is not likely to play a major role in calcium transport and absorption, because only 1% of calcium present in milk is bound to protein (Lonnerdal and Glazier, 1985). Because of its high nutritive value (protein quality), α-lactalbumin plays a major role in providing nutrition to infants and has an amino acid composition similar to the amino acid requirements of newborn infants (Heine et al., 1991; Forsum and Lonnerdal, 1980). α-Lactalbumin is also a part of the lactose synthase enzyme complex, responsible for lactose synthesis in lactocytes. In addition, peptides derived from digestion of α-lactalbumin appear to have antibacterial and immuno-stimulatory functions (Lonnerdal, 2003).

Serum albumin is a major serum protein that is present in human milk and is a source of amino acid for breastfed infants. In addition, leptin a protein hormone involved in regulating body weight is also present in breastmilk at quite a low concentration. This protein could have a contribution in the protective effect of breastfeeding against child obesity by regulating the infant’s food intake (Singhal et al., 2002; Lopez Alvarez, 2007). However, the role of leptin in appetite regulation is complicated because of its association in inflammatory and immunologic pathways (Lopez Alvarez, 2007).

1.5.3.2 Caseins

Caseins are phosphoproteins that precipitate when milk is acidified to pH 4.3 in the presence of calcium. Human milk caseins are composed of different subunits including α (αs1), β and κ-casein; bovine milk contains an additional α-casein subtype (αs2).
Unlike other species, casein in human milk is present in the lowest concentration and predominantly contains β and κ-casein (Lonnerdal and Atkinson, 1995).

The β-casein subunit (24kD) comprises 75% of the total casein and has been shown to be variably modified with 0-5 phosphates (Poth et al., 2008). α₁s-casein (23kD) is the least abundant subunit and also contains phosphorylation sites (Poth et al., 2008). κ-Casein subunit (19kD) is a glycoprotein containing 40-60% of carbohydrates (Lonnerdal and Atkinson, 1995), and comprises less than 15% of total casein (Kunz and Lonnerdal, 1992).

Nearly all casein in milk exists as large colloidal micelle, composed of different subunits, which are bound with calcium phosphate in the centre of the micelle. These large micelles scatter light and give milk its characteristic white colour. It is suggested that the post translation modifications of the casein subunits are important for micelle formation, which is a complex process. It appears that micelle formation is mediated by the attraction between β casein phosphorylated serine residues and charged sialic acid residues of glycosylated κ-casein (Czank et al., 2007d; Lonnerdal and Atkinson, 1995).

Human milk casein micelles are about 30-75nm in diameter and are smaller in size than those found bovine milk (600nm) (Lonnerdal and Atkinson, 1995), which could result in softer and more easily digested curds formed in the acidic environment of infant’s stomach (Lopez Alvarez, 2007).

The complex casein micelle structure can easily be destabilized with change in pH, temperature and by the action of digestive protease (Wong et al., 1996), particularly κ-casein subunit, which is very sensitive to proteolytic cleavage. The cleavage of casein subunits results in release of minerals, essential amino acids and small peptides that
are absorbed by the infant intestine. Thus, casein micelles are the source of the delivery of trace elements (calcium and phosphorus) and amino acids to infants from the mother (Czank et al., 2007d).

In addition to its nutritive role, casein is also a source of bioactive peptides that are released both in the milk (Ferranti et al., 2004) by its intrinsic protease activity and in the stomach during digestion from the stomach proteases such as trypsin (Fiat et al., 1993). Casein-derived bioactive peptides have a multitude of functions including antimicrobial, antihypertensive, antithrombotic, opioid, immuno-modulating and gastrointestinal functions (Clare and Swaisgood, 2000). For example, β-casomorphins are opioid agonist that modulates a number of biological functions such as social and analgesic behaviour, gastrointestinal and endocrine responses (Meisel and FitzGerald, 2000). It is suggested that these peptides produced during breastmilk digestion can be different from those produced during formula or cow’s milk digestion, which may be have an impact on infant nutrition and development (Poth et al., 2008).

1.5.3.3 Milk fat globule membrane proteins

These proteins are associated with the outer surface of the lipid globules in the milk, and represents only 0.3-0.4g/l of total proteins and 1% of fat globule mass (Keenan et al., 1995; Czank et al., 2007d). More than 20 different proteins have been identified in the milk fat globular membrane including butyrophilin, xanthine oxidase, carbonic anhydrase, apolipoproteins, Al, AIV, E and C, mucin 1, lactadherin, fattyacid binding protein etc (Charlwood et al., 2002). Out of which butyrophilin and xanthine oxidoreductase are the most abundant proteins in the milk fat globule membranes. The functional significance of most of these proteins is unknown. However, xanthine oxidoreductase and butyrophilin are responsible for the structural characteristics of
milk fat globules (Keenan et al., 1995). Glycoproteins such as mucins and lactadherin act as ligands for bacteria and viruses and therefore may have immunological properties and protect breastfed infants from infection by microorganisms (Peterson et al., 1998).

Little information is available about the variation in these protein contents. However, it is likely that these proteins remain constant during the course of lactation since fat content of human milk does not vary a lot over time (Lonnerdal, 2003).

1.5.4 Energy

The energy content of human milk can be determined by either bomb calorimetry (Butte et al., 1984b) or calculated by summing the contribution of each of the macronutrient to total energy (Dewey and Lonnerdal, 1983; Nommsen et al., 1991). The reported contributed values of fat, lactose and protein are 37.7 kJ (9 kcal), 16.7 kJ (4 kcal), 16.7 kJ (4 kcal)/g, respectively (Neville and Robert, 1995b) and there is a close relationship between the values determined by bomb calorimeter and calculated energy content (Butte et al., 1984b).

Large variations have been found in the energy content of human milk throughout lactation and in a review of the literature 50% variance was found around a mean value of 293kJ(70kcal)/100 ml, of which 92% is metabolizable energy (Whitehead, 1995; Butte et al., 1984b). Given that the composition of milk is not always constant, it is important that for the determination of energy all levels of variability in composition are taken into account to obtain a representative sample.

A curvilinear relationship reported between energy intake and infant age, with intake decreasing as the infant becomes older, explains the high demand of energy for growth by the newborn infants (Davies, 1998). However, some studies have found
either weak or no relationship between growth and energy intake, rather they found
that growth rate is related more to volume of milk consumed (Dewey et al., 1991a;
Mitoulas et al., 2002). These findings suggest that it is possible that milk intake can be
regulated on the basis of milk composition (Neville and Robert, 1995b).

1.6 Factors affecting milk composition

Human milk composition is not uniform, and depending on the nutrient varies with the
time of the day, stage of lactation and over the course of the feed or expression. The
changes in milk composition may reflect the need of the infant for e.g. higher immune
components during early lactation compared to later stages of lactation. Therefore, in
order to make an appropriate sampling choice to collect a representative sample of all
the milk a mother is secreting, possible variability in composition must be taken in to
account. The factors affecting the composition could either be the maternal
characteristics, time of sample collection, or the gland from which the sample was
taken.

The total protein concentration of human milk declines with the stage of lactation,
decreases rapidly during the first month of lactation and then more slowly during
established lactation (Saarela et al., 2005; Hytten, 1954b; Neville et al., 1991; Bauer
and Gerss, 2011). Longitudinal studies have shown a decrease of around 25% in
protein concentration in the first six months of lactation (Allen et al., 1991; Mitoulas et
al., 2002).

However, there are not only changes in the concentration of total proteins, the
proportion of two main classes of breastmilk proteins, whey and casein, also vary
throughout lactation. The concentration of whey proteins is very high, whereas casein
is almost undetectable during the first days of lactation. Subsequently, casein concentration increases with a simultaneous decrease in the concentration of total whey proteins, partially because of increased milk production (Kunz and Lonnerdal, 1992). As a result of these changes, there is a continuous change in casein to whey ratio in breastmilk during lactation. The ratio of casein: whey has been reported to be low in early lactation (10:90), increased in mature milk (40:60) and levels out at 50:50 in late lactation (Kunz and Lonnerdal, 1992). In addition, changes in the concentration of specific whey proteins have also been reported previously. The protective components of whey proteins (lactoferrin or slgA) are higher in early lactation and subsequently decrease in concentration, whereas the nutritional α-lactalbumin whey protein increases over the course of lactation (Lonnerdal and Adkins, 1999; Sanchez-Pozo et al., 1986). Similarly, the relative proportion of the different β and κ-casein subunits varies as lactation proceeds due to changes in the pattern of posttranslational modifications of their side chains (Kunz and Lonnerdal, 1990a; Kunz and Lonnerdal, 1992). These changes could affect the consistency of curd, which is more solid in mature milk compared to colostrum. This may be related to the physiological capabilities of the infant to digest the milk and maturation of their digestive system during the first weeks of life (Sanchez-Pozo et al., 1986).

In the literature there are conflicting reports about the circadian variation in total protein concentration. Mitoulas et al. (Mitoulas et al., 2002) found no variation in protein concentration over a 24h period, which is in contrast to the finding of Lammi-Keefe (Lammi-Keefe et al., 1990). Furthermore, while there are several studies on longitudinal changes in protein concentration of breastmilk, limited data is available about the change in specific breastmilk components during a feed and over the day.
Peitersen et al. (Peitersen et al., 1975) investigated the immunoglobulin and lysozyme concentrations in breastmilk during a 24hr period and reported no significant change in any of the specific proteins within 24hrs. In contrast, France et al. (Franca et al., 2010) reported a change in immunoglobulins and complement system proteins in the daytime compared to nocturnal period during the first month postpartum. This predominance of immune components in the daytime may be more helpful for an infant’s defence system against infections because there is more chance of contact with infectious agents during the day (Franca et al., 2010).

To our knowledge, there have been no short-term studies (i.e. during the feed and 24h period) investigating the change in both whey and casein composition. This information may help in understanding the control of the milk intake mechanism in infants since differences in the digestibility and absorption kinetics of amino acids from casein and whey proteins could have an impact on infant feeding patterns.

Fat is the most variable of the major energy nutrients in human milk. The total fat content significantly differs between women throughout the day (Mitoulas et al., 2002). There are also marked variations in fat content before (fore milk) and after (hind milk) a breastfeed or expression, with fore milk generally low and hind milk having fat content (Hall, 1979; Neville et al., 1984; Saarela et al., 2005). These variations in fat content in turn influence the energy intake of the infant. The factors that account for the variability in fat content include the volume of milk removed at both pre-feed and current feed, inter-feed intervals and pre-feed fat concentration (Woolridge, 1995). Daly et al. (Daly et al., 1993a) determined that 69% of the variation in fat content of the milk within women was due to degree of fullness of the breast or the volume of milk present in the breast at that time (Cox et al., 1996). They also
showed that an increase in fat content over the course of the feed is related to the
degree of breast emptying i.e. the fat content increases more steeply as the breast is
emptied (Daly et al., 1993a). However, infants who are fed on demand do not empty
the breast to the same degree at each breastfeed and the volume of milk consumed
does not necessarily result in complete emptying of breast. It has been shown that on
average, the baby consumes only 67% of the available milk (Kent et al., 2006). This
suggests that the infant feeding pattern may be responsible for the circadian rhythm
observed in the fat concentration (Daly et al., 1993b). Longitudinal changes were also
observed in fat concentration, with the reports of a decrease in fat content at 2
months and then increasing again at 9 month over the 12 months of lactation
(Mitoulas et al., 2002; Allen et al., 1991; Butte et al., 1984a).
Lactose tends to be the least variable macronutrient in human milk, with no diurnal
and within feed changes observed in lactose concentration (Mitoulas et al., 2002;
Neville et al., 1984). However, a significant increase is observed in lactose
concentration over the first six months of lactation (Allen et al., 1991; Neville et al.,
1991). In established lactation, milk production responds to the infant demand for milk
and it is presumed to be under autocrine control, which in combination with endocrine
changes may result in short and long term differences in milk composition. Despite the
reports of acute changes in milk composition in established lactation (Hartmann and
Prosser, 1984) there are limited studies that have investigated the week-to-week
variation in milk composition during this period.
1.7 Infant feeding patterns

There is no prescribed feeding pattern for breastfeeding infants but the current recommendation for mothers is to breastfed their infants “on demand,” according to their appetite (Kent, 2007). Marked variations have been reported in the frequency of breastfeeding and volume taken at each breastfeed both within and between mother-infant pairs throughout the day (Butte et al., 1984b; Kent et al., 2006; Dewey and Lonnerdal, 1983). A recent study carried out in an Australian group of mother-infant dyads indicated a breastfeeding frequency of 11±3 times, ranging from 6-18 in 24h, with a breastmilk intake of 76±13g per meal (Kent et al., 2006). Breastfed infants also vary in the amount they take per day with reported ranges of 500-1300ml/day and in their patterns of milk intake (Kent et al., 2006). Some infants breastfeed at night as well, which may be due to their small stomach storage capacity and/or faster gastric emptying rate (Kent et al., 2006). Moreover, larger breast feeds in the morning and lower intake in the evening were also observed in breastfed babies (Butte et al., 1985). These variations in feeding patterns, combined with the high variability in breastmilk composition and energy content (Mitoulas et al., 2000; Whitehead, 1995) account for great variability in energy intake between infants and thus the infant growth. It has been shown that growth rate is more strongly related to the volume of milk consumed rather than the energy intake (Dewey et al., 1991a; Mitoulas et al., 2002). However, the factors that are involved in this regulation of milk intake are not clearly understood.

1.7.1 Self-regulation of breastmilk intake

There is strong evidence in the literature that breastfed babies determine their milk intake. For example, the measurement of breast volume before and after each
breastfeed over the 24h period has shown that infants do not drain the breast at each feed (Daly et al., 1993b) and on average, consume 67% of the available milk in the breast. This indicates that infants self-regulate their intake of breastmilk; they do not stop feeding because the breast is empty but feed according to their appetite (Kent et al., 2006).

Similarly, Dewy et al. (Dewey and Lonnerdal, 1986) has observed an increase in milk supply of mothers when they were asked to express extra milk after the usual breastfeeds. This shows that mothers have the potential to increase their milk production and milk intake was not limited to maternal milk yield and was primarily determined by the infant demand or appetite. In addition, Ramsay et al. (Ramsay et al., 2004) found that infants are able to terminate the breastfeed during a milk ejection, which also again suggest that the baby is controlling its milk intake, rather than being limited by the number of milk ejections or milk flow.

There are large numbers of potential factors that may influence the milk intake in infants such as milk composition or the infant’s stomach storage capacities and/or gastric emptying rates or a combination of both. However, limited data is available in this regard, with only few reports of a relationship between infant weight and milk intake (Dewey et al., 1991b).

1.8 Gastric emptying (GE)

Successful infant feeding requires effective coordination of suckling, swallowing, gastric emptying and intestinal motility and a problem at any level has the potential to effect the infant feeding behaviour (Veereman-Wauters, 1996). Gastric emptying is the process in which food is evacuated from the stomach into the small intestine for
further digestion and absorption; therefore this process has a metabolic impact. It is considered as a key regulator of appetite since rapid gastric emptying can lead to a much earlier sensation of hunger (Hunt, 1980) and also has a number of important health implications for infants. For example: delayed GE may result in feed intolerance and gastro-esophageal reflux, which is common in term and preterm infants. It presents as failure to tolerate milk feeds and can result in vomiting (Fonkalsrud and Ament, 1996). In addition, it has been suggested that altered GE rates may have a role in colicky behaviour in infants (Gupta, 2002).

1.8.1 Physiology and Control of GE

In infants, gastric emptying seems appropriately developed from 32 weeks on (Veereman-Wauters, 1996), but information on the physiology of gastric emptying and normal motility patterns in paediatrics is still limited. However, based on adult physiology, gastric emptying of a meal is accomplished by the coordinated activity of the proximal and distal regions of the stomach to empty the different phases of the meal into the small intestine in an appropriate form and at an appropriate rate for further digestion and absorption (Horowitz et al., 1994). The stomach is divided into three sections; fundus, body (or corpus) and pylorus. The fundus is proximal and lies above the level of esophageal opening. The body includes the middle and distal portion of the stomach, which known as antrum and lies above the pyloric sphincter. After meal ingestion, the proximal stomach relaxes so that the meal can be accommodated. The distal stomach grinds the food into small particles and propels the resultant chyme into the duodenum against pyloric resistance. The presence of nutrients in the small intestine generates neural and hormonal feedback on gastric motor function. This feedback slows the further emptying to a closely regulated rate
by relaxing the proximal stomach, inhibiting antral contractions, and stimulating tonic and phasic pyloric pressures (Figure 1.1)(Rayner and Horowitz, 2005). Thus, gastric emptying is controlled by metabolic (blood sugar), neuronal and hormonal signals, mainly acting to slow the emptying process after food intake (Hellstrom et al., 2006). Gut-derived hormones and transmitters, which are released in response to nutrients influence appetite and subsequent energy intake through central or peripheral actions. Gut hormones include ghrelin, insulin, GIP (glucose-dependent insulinotropic peptide), cholecystokinin (CCK), glucagon like peptide (GLP-1), oxyntomodulin, pancreatic peptide (PP) and peptide YY (PYY)(Hellstrom et al., 2006; Camilleri, 2009). However, in addition to the gastric emptying and gut hormones other interacting mechanisms are also involved in the appetite control of adults. These include the Hypothalamic peptidergic circuits that express appetite controlling receptors such as cannabinoid CB1 and neuropeptide Y (NPYN), and adipose tissue derived hormones such as leptin (Camilleri, 2009; Simpson et al., 2009).
Figure 1.1: Motor events associated with normal gastric emptying.

The fundus (proximal stomach) relaxes to accommodate the meal, while antrum (distal stomach) grinds the solids and propels the resultant chyme into duodenum against the pyloric contractions. The presence of nutrients in the small intestine produce neurohormonal feedback that delays further emptying by stimulating the fundus relaxation and pyloric contractions, while reducing the antrum motility (Adapted from Rayer and Horowitz, 2005)(Rayner and Horowitz, 2005).

1.8.2 Patterns and rates of GE in infants

The rate and patterns of gastric emptying are dependent upon the nature (i.e. liquid or solid) and the macronutrient composition of the ingested meal. Solids empty from the stomach after an initial lag phase in which there is minimal emptying, this phase is then followed by a roughly linear phase of emptying. In the case of liquids, nutrient
containing liquids (e.g. juices) and liquefied solids (e.g. cereals) empty from the stomach in a linear manner, whereas non-nutrient liquids (e.g. water) empty from the stomach in a mono-exponential fashion (Horowitz and Dent, 1991) (Figure 1.2).

![Figure 1.2: Patterns of emptying of solid and liquid meal.](image)

[Adapted from Bowen 2005 (Bowen, 2005)].

In contrast to adults, infants ingest only liquids either in the form of breastmilk or formula. But milk ingested by babies separates in the stomach into two phases, a semi solid phase consisting of curd formed by casein and a liquid phase consisting of water, whey proteins, lactose, and other elements. The semi solid phase of the milk is digested slowly whereas the fluid portion is emptied rapidly from the intestine (Mahe et al., 1996). The slow emptying of this solid component of milk (curd) from the stomach may have a clinical significance in the management of colicky babies. Different emptying patterns have been reported not only between breastmilk and
formula but also for breastmilk in infants. Cavell (Cavell, 1979; Cavell, 1981), investigated the gastric emptying of infant formula and breastmilk in full term and premature infants and observed a biphasic-emptying pattern with an initial fast phase after meals of human milk whereas infant formula followed either a linear pattern or showed an initial delay of gastric emptying. However, Gomes et al. (Gomes et al., 2003) have identified different types of emptying patterns for breastmilk including a monophasic linear pattern and a biphasic pattern with an initial rapid emptying phase followed by a slower phase. These differences in patterns could be due to the differences in milk composition, which is not clear.

The gastric emptying half time ($t_{1/2}$), which is a statistically defined parameter, is most consistently used to report gastric emptying in adults and infants. Although Gastric emptying rates reported in the literature are variable depending on the technology used, physiological gastric emptying of breastfed infants is faster than gastric evacuation of infants receiving formula. The average reported $t_{1/2}$ values for breast milk are 48, 36 and 47 min whereas for formula the reported halftime was 78, 72 and 65 min with marker dilution, ultrasound and breath test techniques, respectively (Cavell, 1981; Ewer et al., 1994; Van Den Driessche et al., 1999).

1.9 Factors affecting GE

The key factors that influence gastric emptying in infants are the volume and the composition of the feed (Siegel et al., 1985; Hunt and Stubbs, 1975; Salvia et al., 2001). Furthermore, not only the concentration but also the type of macronutrient and their degree of hydrolysis play an important role in gastric emptying. For example, ingestion of large native fat globules result in faster gastric emptying (GE) when compared to
small homogenized globules in human adults and small animal models (Michalski et al., 2005a) and each type of protein has its own emptying rate (Khoshoo and Brown, 2002).

Other factors including posture, activity, appetite, pathology (presence of gastro-esophageal reflux), psychomotor development and individual factors have also been shown to influence gastric emptying (Arienti et al., 1994; Fonkalsrud and Ament, 1996; Brown et al., 1994; Ramirez et al., 2006).

1.9.1 Milk Composition

Since human milk composition varies both within and between women and the composition (concentration of energy yielding macronutrients) and volume of milk produced are influenced by a number of factors. It is likely that changes or differences in the nutrient content of breastmilk may have an influence on the rate of gastric emptying which could have an affect on feeding behaviour of infants (Lorenz, 1985).

Human milk has a unique composition, which differs from that of other mammals in its components and their concentrations. Because of this unique composition none of the current formula preparations are comparable to human milk and do not provide the same mixture of components present in human milk. Most of the studies that investigate the effect of macronutrient composition on gastric emptying in infants are based on formula milk. These studies have reported that factors such as high fat, carbohydrate, protein content, high energy density and high osmolarity of formula resulted in delayed gastric emptying (Siegel et al., 1985; Salvia et al., 2001). However, there is no information whether similar relationships of the different nutrients to gastric emptying are present for breastmilk or not.
Gastric emptying studies have shown that breastmilk empties at a faster rate than formula milk indicating that breastmilk is more quickly digested and absorbed by infant’s immature digestive system (Tomomasa et al., 1987). When infants were fed with equal volumes (50ml) of expressed breastmilk and formula, breastmilk emptied around 20min (on average) faster than formula milk and this difference was statistically significant (Van Den Driessche et al., 1999). It has also been demonstrated that in breastfed neonates interdigestive (fasting) state appeared more quickly than the formula fed infants (Tomomasa et al., 1987). Further more, fewer signs of hunger have been observed in infants after ingestion of formula milk probably because it remains in the stomach longer than mother’s milk (Tomomasa et al., 1987; Hartmann PE, 1991).

Although there are several differences between breastmilk and formula milk, a possible reason for the variation in their gastric emptying may be the protein composition, which from the literature is known to be an important factor influencing gastric emptying since the behaviour of caseins and milk water-soluble proteins differ markedly during digestion in the stomach (Fried et al., 1992). Further more, dietary proteins have been shown to be more suppressive in short-term food intake and have higher satiety effect than either carbohydrates or fats in both animals and humans (Anderson and Moore, 2004).

1.9.2 Casein Curding

Substantial differences exist between breast and bovine milk protein composition, which is the base of most infant formula. Bovine milk not only has higher protein concentration (range 15-17g/l) but also has different whey to casein ratio and different casein subunits (Sherriff and Hartmann, 2000). Whey and casein are present in a ratio
of 60:40 in breastmilk (Kunz and Lonnerdal, 1992), whereas in Cow’s milk (the base of most infant formula) the ratio of whey to casein is 20:80 and variation in this ratio has been shown to affect GE (Khoshoo and Brown, 2002). It has been shown in term neonates that whey predominant formulae emptied faster than casein predominant formulae indicating that casein is more effective in slowing down gastric emptying (Billeaud et al., 1990). In addition, slower gastric emptying is seen in milk that contains more intact casein when compared with acidified formula (Billeaud et al., 1990), which suggest that acidification or hydrolysis of casein in milk may facilitate digestion in infants (Ferranti et al., 2004). Milk ingested by the infants separates into successive layers of curd and fluid portion in the stomach. The curd formed is due to precipitation of the casein under the acidic gastric pH which delays it’s emptying whereas milk water-soluble proteins are rapidly evacuated from the stomach. When compared with bovine milk, breastmilk (because of its low casein content) forms soft flocculent curd in the stomach of the infant, which is consistent with the high frequency of feeding, observed in breastfed infants (Hartmann PE, 1991).

1.9.3 Feed Volume

Gastric distension has also been involved in the regulation of appetite and energy intake (Lorenz, 1985). The volume of milk in the stomach varies throughout the day and it was shown in rats that variations in gastric volume is involved in controlling the gastric emptying rate of milk and is also correlated with the suppression of ingestion in rat pups (Lorenz, 1985). This phenomenon may be present in human infants as well, since volume of ingested breastmilk differs from one feed to another among individual infants (Hibberd et al., 1982; Kent et al., 2006). There is very limited data available regarding the importance of gastric volume in the control of gastric emptying in infants.
particularly in term infants. Based on adult studies, it is unlikely that gastric distension alone acts as a signal that can terminate milk intake, as generally emptying of liquid is dependent on its energy density. The higher the energy density the slower is the emptying (Horowitz and Dent, 1991). However, unlike adults, it has been shown in preterm infants that altering osmolality, volume or energy density do not individually alter GE. In contrast, simultaneous reduction of osmolality and increasing feeding volume has been found to increase GE in these infants (Ramirez et al., 2006). Moreover, in breastfed term infants volume of breastmilk intake has not been investigated as a factor contributing to gastric emptying.

1.10 Methods for assessing GE

Numerous methods have been used for assessment of gastric emptying including dye dilution techniques, radiolabeled scintigraphy, stable isotope breath test, magnetic resonance imaging (MRI) and ultrasonography. Radioscintigraphy is considered the gold standard for estimating gastric emptying in clinical practices in adults. This technique involves the ingestion of radiolabelled meal followed by the scanning of gastric area at different intervals with an external gamma camera to obtain stomach images for quantification of gastric emptying (Szarka and Camilleri, 2009). However the technique is inappropriate for use in infants because of the radiation exposure and the requirement to keep infant immobilized during testing under a gamma camera (Hveem et al., 1996).

Other techniques that have been used previously in preterm infants are marker dilution and sampling techniques. These techniques involve the repeated withdrawal, reinsertion and mixing of gastric contents to measure the gastric volume, which is not
representative of the physiological process, and is not well tolerated by smaller infants (Husband and Husband, 1969; George, 1968). Also these techniques are not able to show the pattern of emptying for an individual feed. These methods are invasive, impractical and difficult to perform and are not suitable for use in infants.

Current non-invasive techniques for the assessment of gastric emptying in infants involve ultrasound scanning of the gastric antrum, magnetic resonance imaging (MRI) and $^{13}$C-Octanoic acid breath testing. The breath test measures the oxidation of $^{13}$C-Octanoic acid into $^{13}$CO$_2$ and uses the rate with which $^{13}$CO$_2$ appears in the breath to calculate the emptying rate of the meal or liquid (Barnett et al., 1999). Although this test is a feasible, reproducible and non-invasive and can also be used to compare different feeding methods (Van Den Driessche et al., 1999), imaging techniques have an extra advantage over this method. Imaging techniques like ultrasonography and MRI have the capacity to directly measure what is happening to the meal in the stomach (such as curdling of milk) in a non-invasive manner (Szarka and Camilleri, 2009). Of the imaging techniques ultrasonography has been most widely applied to measure GE in infants. MRI is sensitive to patient movements and requires sedation in the neonates and also requires the use of extremely expensive equipment, which restricts its use for research purposes (Szarka and Camilleri, 2009).

Ultrasound imaging being reproducible, non-invasive and inexpensive is an ideal assessment method to monitor gastric emptying in infants. This method has been previously used successfully both in adults and infants and has not been shown to have any side effects (Bolondi et al., 1985; Newell et al., 1993).

The basic principle of the technique is that when short pulses of ultrasound pass through a liquid and are partially reflected by solid interfaces, they produce echoes.
The depth of the reflecting interface determines the time taken for an echo to return to a transducer on the skin. The transducers are rapidly activated in turn to produce a continuous, moving, two-dimensional image of a slice of a tissue, such as a cross section of stomach. The volume of the stomach can thus be computed from the measurements of the areas of the cross-sectional images (Bateman, 1982b).

The most commonly used ultrasound-imaging method to evaluate gastric emptying in preterm infants is the measurement of antral cross section area (ACSA) of the stomach over time. This technique rather than directly measuring the gastric emptying measures the clearance of the feed through the antrum and therefore is a proxy for gastric emptying (Newell et al., 1993). Newell et al. has validated the technique by measuring the ACSA after administration of 25, 50, 75 and 100% of the feed volume in preterm infants and demonstrated the linear relationship between the feed volume and ACSA (r=0.78)(Newell et al., 1993). This technique measures only a small portion of the stomach, which limits the possibility of gaining extra information by imaging. In contrast, ultrasound direct measurement stomach volume technique involves scanning of the stomach in its entirety, and has the potential to provide information about intra-gastric curding. This technique has previously been used in term infants (Lambrecht et al., 1988) but requires validation to evaluate gastric emptying in breastfed infants.

In addition since limited research has been undertaken on gastric emptying of healthy term breastfed infants, it is likely that variation in the composition and volume of breastmilk have an effect on patterns of gastric emptying in these infants. Therefore, it is important to investigate the effect of milk constituents on gastric emptying along with gastric emptying profiles, as this would help in understanding the regulation of
food intake in infants and in addition provide guidance for the transitioning of babies from breastfeeding to solid foods.

1.11 Research Aims

The project was set out to understand the factors involved in the regulation of milk intake in infants by investigating both maternal factors including variation in milk composition and production, and infant related factors including stomach storage capacity and gastric emptying rate in healthy term infants. This information would help in understanding the physiology of gastric emptying in term infants.

Therefore, the aims of this research project were:

- To investigate the circadian and within expression variations in casein and whey proteins in breastmilk.
- To investigate the short-term (weekly) variations in fat, protein, lactose, total solid, and energy content of breastmilk.
- To investigate the influence of variation in fat, lactose and protein composition of breastmilk over 24h on infant feeding patterns.
- To validate and assess the reliability of ultrasonic measurements of infant stomach volume to monitor gastric emptying.
- To assess the relationship between gastric emptying and both the composition and volume of a breastfeed in term infants.
CHAPTER 2  Investigation of short-term variations in casein and whey proteins in breastmilk of term mothers

Sadaf Khan¹, Ylenia S Casadio¹, Ching T Lai¹, Danielle K Prime¹, Anna R Hepworth¹, Naomi J Trengove¹, Peter E Hartmann¹

¹School of Chemistry and Biochemistry, The University of Western Australia, Crawley, Western Australia, Australia

2.1 ABSTRACT

Objectives – We investigated changes in breastmilk whey and casein proteins, between fore- and hind-milk during breast expression, between breasts and over 24hr period during breastfeeding. This has implications for developing appropriate sampling protocol for investigating the influence of milk composition on gastric emptying and infant’s feeding behaviour.

Methods - Breastmilk samples were collected from mothers (n=25) of healthy term infants aged 1 to 8 months. 17 mothers provided fore- and hind-milk samples, which were collected during simultaneous expression of both breasts. 15 mothers provided samples from each breastfeed over 24hr period, of which samples were selected from four time points (morning, day, evening and night). Whey and casein were isolated from skim milk, and protein concentration of the skim, whey and casein fractions were determined.
Results - Mean protein concentrations were found to be 13.5±2.1g/l (skim milk), 7.6±1.5g/l (whey) and 3.4±0.97g/l (casein). Protein concentrations were not significantly different between the fore- and hind-milk. Over 24hr period, no significant differences were found in protein concentration of any fraction at the four time points or between left and right breasts. Large variations were seen between mothers with CVs of 15.5%, 19.8% and 28.4% for skim milk, whey and casein, respectively.

Conclusion – While there was wide variation between mothers, the small variations within mothers indicates that for sampling purposes a single breastmilk sample (fore or hind from each breast at any time point of the day) will be representative of that mother’s protein concentration of skim, whey and casein fractions for that day.

2.2 INTRODUCTION

Breastmilk proteins provide both nutritional and physiological benefits to infants and longitudinal, circadian and individual variations have been reported to influence the breast milk protein concentration (Kunz et al., 1999; Mitoulas et al., 2002; Allen et al., 1991; Clark et al., 1987; Lammi-Keefe et al., 1990; Michaelsen et al., 1990). While there are several studies on longitudinal changes in total protein concentration of breastmilk, information on the circadian variations, in particular variation in the two main classes of breastmilk proteins whey and casein is limited. Furthermore, to our knowledge there have been no short-term studies (within the day) investigating the variability in both whey and casein composition.

It is important to study the short-term variations in individual protein concentrations as this information has implications for appropriate sample collection of these constituents for future nutritional studies. This information may also help in
understanding the control of the milk intake mechanism in infants since protein composition has been reported as an essential factor contributing to gastric emptying. Casein and whey proteins behave differently during digestion in the stomach and have different emptying rates (Khoshoo and Brown, 2002), and therefore could contribute to variations in infant feeding patterns.

Previous studies have shown that there are not only changes in the total protein concentration but also the proportion of casein and whey proteins vary over the course of lactation in both term (Kunz and Lonnerdal, 1992) and preterm (Sanchez-Hidalgo et al., 1998) human milk. While total protein concentration decreases as lactation progresses, casein concentration, which is almost undetectable during the first days of lactation, increases with a simultaneous decrease in the concentration of total whey proteins. Due to these changes, the ratio of casein to whey proteins in breastmilk has been found to vary throughout lactation (i.e. from initiation to up to 12 months of lactation) and reported to be low in early lactation (10:90), increased in mature milk (40:60) and levels out at 50:50 in late lactation (Kunz and Lonnerdal, 1992). Since it is known that there are longitudinal changes in the relative concentration of casein and whey proteins during lactation, further investigation is required to understand whether the casein: whey ratio varies over the short term, i.e. throughout the day.

This study was designed to investigate the variation in protein concentration of breastmilk casein and whey fractions in mothers of term infants. More specifically, we aimed to investigate whether the protein concentrations differed between fore and hind milk, between breasts of mothers, over four time points within a 24 hour period as well as investigating the extent of variation between mothers.
2.3 MATERIALS AND METHODS

2.3.1 Subjects

Breastmilk samples were obtained from twenty-five mothers of healthy, term singleton infants recruited through the Western Australian branch of the Australian Breastfeeding Association, and through Child and Adolescent Community Health Nurses in the Oceanic Health Region. The infants (n=25) had a mean age of 3.6±1.9 months (range 1.0-8.0 months) and were mostly male (n=18). All infants (n=25) were exclusively breastfed on demand except for two infants older than six months who were receiving complementary solid foods. The infants were growing appropriately for age. Briefly, mothers were between 25 and 39 years of age, (mean 33±4.0 years, n=4 age unknown) and majority of them were primiparous (n=16). Seventeen mothers (mean age 33±3 years) agreed to provide the samples for the analysis of protein concentration in fore and hind milk samples (Study component A, mean age of infants 3.7±2.1, range 1-8 month, % male infants 82%). Fifteen mothers (mean age 31±4 years) including a subgroup of 7 mothers from Study component A provided the samples with sufficient volume for the analysis of circadian variation in protein concentration over a 24hr breastfeeding period (Study component B, mean age of infants 3.2±1.7, range 1-6 month, % male infants 67%). Mothers were also asked to measure their 24h milk productions.

All mothers provided the written informed consent to participate in the study, which was approved by The University of Western Australia, Human Research Ethics Committee.
2.3.2 Sampling

**Study Component A:** Mothers (n=17) performed a 15-minute simultaneous expression of left and right breasts using two separate electric breast pumps (Symphony, Medela AG, Switzerland). This was done under supervision and milk samples (1-2ml) were collected at the beginning (fore) and end (hind) of the expression session by the supervising researcher. Total volume of milk expressed during the session was recorded. Subjects who participated in this part of the study attended our experimental rooms at the Breastfeeding Centre at King Edward Memorial Hospital, Subiaco, Western Australia.

**Study Component B:** Mothers (n=15) collected the milk samples (1-2ml) at their homes before and after each feed from each breast over a 24hr period by either hand expression or manual hand pump into 5 ml polypropylene vials (Disposable Products Pty Ltd, Adelaide, Australia). For this study, the day was divided into four intervals of six hours and named as morning (4:01AM to 10:00AM), day (10:01AM to 4:00 PM), evening (4:01PM to 10:00PM) and night (10:01PM to 4:00AM) as described previously by Kent *et al.* (Kent *et al.*, 2006).

The samples collected before and after the feed were pooled. Then milk samples from left and right breast that were closest to the centre of these time points were selected for the analysis of the concentration of proteins.

Samples were initially stored in the mother’s home freezer and then transported on ice to the laboratory where they were kept at -20°C until further analysis.

2.3.3 24h Milk production measurements

Mothers measured their 24hr milk productions at their homes by test weighing the infants before and after each feed, for each breast, using electronic scales (Baby Weigh
Scale, Medela AG, Switzerland) and recorded the volume of milk intake of each feed from each breast (Arthur et al., 1987).

2.3.4 Isolation of casein and whey

The milk samples were thawed and after mixing defatted by centrifugation at 10,000 x 
g for 10 min at 4°C for the analysis of concentration of proteins. Casein and whey proteins were separated by the method described by Kunz and Lonnerdal (Kunz and Lonnerdal, 1989; Kunz and Lonnerdal, 1990b). In short, casein and whey were separated from the defatted (skim) milk by addition of calcium chloride to the skim milk, pH was adjusted to 4.3 with 1M HCl followed by ultracentrifugation at 189,000 x 
g for 90 min at 4°C. The clear supernatant containing whey was separated and the casein pellet was washed with double deionised water and centrifuged again at 189,000 xg for 60 min. The supernatant wash was collected and the casein pellet was dissolved in surfactant solution of 6M urea and 4% 3-((3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid (CHAPS). The samples were stored in -80°C freezer until analysed.

The separated fractions were subjected to electrophoresis in 15% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) (Laemmli, 1970). In the electrophoresis purified human milk β-casein, lactoferrin and serum albumin (Sigma-Aldrich, NSW, Australia) were included to control for sample purity. Protein bands were identified by comparing to those of purified human milk proteins and with protein standards of known molecular weights varying between 10 and 250 kD (Precision Plus Protein Standards, Bio-Rad).
**2.3.5 Protein assay**

The protein concentrations of skim, whey, casein and casein wash fractions of the samples were determined by the Bradford protein assay using a commercial protein reagent (Bio-Rad Laboratories, Richmond, CA, USA). Human milk protein standards were prepared by determining the concentration of an aliquot of mature breastmilk as described by Atwood and Hartmann (Atwood and Hartmann, 1992). The protein assays were carried out by the procedure described by Mitoulas et al. (Mitoulas et al., 2002). Skim milk and whey samples were diluted 1 in 30, for casein samples 1 in 10 dilution was used. The protein concentration of casein samples was measured by comparing to protein standards that were prepared in the same surfactant solution used to dissolve the casein pellet in order to avoid any background interference. For all assays a quality control sample was analysed along with all the samples. The recovery of a known amount of the protein added to the milk samples was 99.8% (standard error [SE] 1.4, n=12). The detection limit of the assay was 0.045 g/l (SE 0.002, n=30) and the inter-assay coefficient of variation was 6.4% (n=50).

**2.3.6 Statistical Analysis**

Statistical analysis was performed using the R program, version 2.7.2 (R Development Core Team, 2009). The package nlme (Pinheiro J et al., 2009) was used for linear mixed effects modelling. For the linear mixed models, different intercepts for each individual were used as the random effect in all models, unless otherwise specified. Descriptive statistics are reported as mean±standard deviation, unless otherwise stated. P values less than 0.05 were considered statistically significant.

**Fore and hind milk:** Protein concentrations of fore and hind milk samples were compared using univariate linear mixed effect models that tested the calculated
difference against zero (intercept only model). Expression volume was tested as univariate predictor.

**Circadian variations:** Linear mixed effect models were used to compare the circadian variation in protein concentrations by considering time of day as a factor. Age of infant and feed volume were tested as univariate predictors, and where significant relationships were seen they were included as covariates in the time point model for that protein fraction. Models presented include only significant covariates. Infant age was included either as linear predictor, or stratified into younger (≤ 2months) and older (≥ 3months).

**Comparison of breasts:** Comparison of average feed volumes and 24h milk intakes between breasts used paired Student's t-test. Individual differences in baseline protein concentrations between the breasts were tested by including breast as a random effect in circadian variation models.

The influence of mechanical expression on protein composition was assessed in a sub group of seven mothers who participated in both components of the study, using coefficient of variance as a measure of magnitude.
2.4 RESULTS

2.4.1 Participants

Mothers (n= 25) participating in all components of this study completed the 24h milk production in order to measure the feeding patterns of the infants and had no concerns about their lactation.

The mean 24hr breastmilk intake was 758±162 g (range 467-1113g) and there was no significant difference between the 24hr milk intake from left (368±133g, range 133 to 665g) and right breast (372±128g, range 85 to 598g). Breastfeeding frequency (a continuous sucking session from one breast (Kent et al., 2006)) ranged from 6-21 feeds/24hr (mean 14±3). The average breastfeed volume was not significantly different between the left (62±22 g) and right (60±22 g) breasts.

The milk intake patterns were within the range reported previously for these measurements (Kent et al., 2006).

2.4.2 Separation of casein and whey

Figure 2.1 depicts the skim milk, separated whey and casein fractions. While it is known to be difficult to completely separate the whey and casein proteins (Sanchez-Hidalgo et al., 1998), it can be seen that the casein band was not predominantly present in the separated whey and vice versa. Therefore, the “acid-soluble fraction” was defined as whey and “acid- insoluble fraction” as casein.
Figure 2.1: Gel electrophoresis of separated skim milk, whey and casein fractions. Lanes at the top showing MW, reference molecular weight markers; Skim, human milk proteins from skim milk; Whey, separated whey and Casein, casein fractions; β-Casein, human milk β-casein standard; whey protein standards; Albumin, serum albumin and Lactoferrin.

2.4.3 Protein concentration in fore and hind milk

Study component A examined the protein concentrations in fore and hind milk from a single breast expression for 17 mothers. This data is presented in Figure 2.2. Overall, the mean protein concentrations were 12.85±2.2g/l for skim milk, 7.06±1.85g/l for whey and 2.99±0.73g/l for casein. The concentration of protein was not significantly different between the fore and hind milk samples in the skim (mean difference=−0.12g/l, p=0.78), whey (mean difference=−0.06g/l, p=0.89) and casein (mean difference=−0.09,p=0.23) fractions.

A negligible amount of protein (0.52±0.19g/l) was present in the casein wash, which was found to be predominately casein based on gel analysis. The difference between
the fore and hind milk casein wash values (mean difference=0.01 g/l, p=0.82) were also not significantly different. Therefore, for analysis purposes the casein and casein wash values were summed together.

In addition, the total volume of milk expressed was not found to affect the protein concentration of any fraction.

**Figure 2.2:** Distribution of protein concentration of skim, whey and casein fractions in fore and hind milk collected during breast expression. Values are shown by box plots illustrating median (indicated by the bold line), quartiles (box), range (error bars) and outliers (o) (n=17). No significant differences were found.
2.4.4 Circadian and Inter-individual variation in protein concentration

Study component B analysed the protein concentration at the four time periods (morning, day, evening and night) within 24hr for 15 mothers. This data is presented in Figure 2.3. The average protein concentrations in this data set for all mothers were found to be 13.55±2.1g/l, 7.6±1.5g/l and 3.4±0.97g/l for skim milk, whey and casein respectively.

The total protein concentration in skim milk samples was similar at all time points in the day (p=0.14). There was a non-significant (p=0.055) trend towards a higher protein concentration in the evening samples; with 0.67g/l (5%) more protein measured when compared to morning. Neither whey protein concentration (p=0.55) nor the casein protein concentration (p=0.18) was found to be significantly affected by the time of the day.

While the average protein concentration for all mothers in each of the fractions remained constant throughout the day there were marked variations observed between individual mothers. The protein concentration in skim milk, whey and casein fraction ranged from 7 to 18.1 g/l (CV 15.5%), 4.45 to 11.35 g/l (CV19.8%) and 1.66 to 5.26 g/l (CV 28.4%), respectively, between mothers.

When compared to skim milk, the variation in whey and casein fractions was more pronounced both within and between mothers. Analysis of the Coefficient of Variance (CV) of the protein concentration for samples from individual mothers showed that for the whey fraction, eight mothers and for the casein fraction six mothers had a CV greater than 10% during the day. However, while variation above 10% existed (11.7-18.5% whey; 13.1-26.8% casein) the pattern was not consistent with a circadian variation.
No relationship was seen between the volume of milk consumed by the infant during the feed (feed volume) and the protein level in the skim (p=0.83), whey (0.08) and casein fractions (0.09).

Protein concentration of the whey fraction was found to decrease significantly with the age of the baby (p=0.03), while the effect was not significant for the skim milk and casein fractions. In addition, there was no relationship between infant age (either in months, or stratified into younger and older infants) and protein concentration of any fraction at any time point of the day.

There was a significant relationship between the protein content of skim milk to whey and casein fraction (p<0.05). Higher protein concentration of skim milk was associated with higher concentrations of both whey and casein fraction.
Figure 2.3: Distribution of protein content of skim milk, whey and casein fraction of milk samples taken during breastfeeding in the morning, day, evening and night. Values are shown by box plots illustrating median (bold line), quartiles (box), range (error bars) and outliers (o) (n=15). No significant differences were found.

2.4.5 Protein concentration between breasts

There was no overall difference in the protein concentration of skim (p=0.09), whey (p=0.054) and casein fractions (p=0.14) between breasts (Figure 2.4). Similarly, no consistent differences were detected in the protein levels between breasts across selected time points of the day. However, individual variations were found in three mothers who had consistently different whey protein concentrations between their breasts.
When mean protein composition of samples obtained from breast expression were compared to the hand expressed breastfeed samples in a subgroup of 7 mothers, the variability was under 10%.

![Figure 2.4: Comparison of protein concentration between breasts in skim, whey and casein fractions across four time points of the day.](image)

Box plots representing median (indicated by the bold line), quartiles (box), range (error bars) and outliers (o). Day was divided into six four hour intervals named as morning, day, evening and night. No significant differences were detected.

### 2.5 DISCUSSION

This study measured the short-term changes in protein composition of term human milk. Within mothers the protein concentration of skim, whey and casein fractions over a 24hr period did not vary between fore and hind milk samples or the left and
right breasts (Figure 2.2 and Figure 2.4). There was, however, considerable variation between mothers.

Our results of no significant change in fore and hind milk total protein concentration (Figure 2.2) are in agreement with other previous studies carried out both in women (Mitoulas et al., 2002; Saarela et al., 2005) and other species (Atwood and Hartmann, 1992). Furthermore, no circadian change was found in protein concentration of any fraction; with no difference between morning, day, evening and night feed samples (Figure 2.3). This finding supports earlier work of Mitoulas et al. (Mitoulas et al., 2002). However, Lammi-Keffe et al. (Lammi-Keefe et al., 1990) found a significant time of the day effect on protein nitrogen. The change observed in the study by Lammi-Keffe et al. could be associated with the change in fat concentration over the day, as it appears that a whole milk sample was used for protein analysis.

During lactation, protein composition changes to meet the growing requirements of the infant (Lonnerdal, 2003). A decrease in whey protein concentration is balanced by a subsequent increase in casein concentration (Kunz and Lonnerdal, 1992). Changes in the concentration of specific whey proteins have also been reported previously. The protective components of whey proteins (lactoferrin or sIgA) are higher in early lactation and subsequently decrease in concentration, whereas the nutritional alpha-lactalbumin whey protein increases over the course of lactation (CuilliÈre et al., 1997). However, we did not find any short-term variations in the relative proportion of casein and whey proteins.

Our finding of no consistent change in protein concentration between breasts is supported by previous studies (Mitoulas et al., 2002; Neville et al., 1984)(Figure 2.4). However, we did observe consistent differences in protein concentration particularly
in the whey fraction of some individuals. These mothers (n=3) had consistently higher protein concentration (approximately 1.5g/l) in one breast than the other for all samples collected over 24hr period. Of these individuals, two had asymmetrical productions, with one breast producing around 40% of the milk production of the other. These were older mothers (ages 33 and 37 years). The third individual did not show a difference in production between breasts, and her age was unknown. Neville et al. (Neville et al., 1984) has observed the inconsistent differences in the milk composition of micronutrients between left and right breasts and suggested that mastitis might contribute to these differences. The mothers participating in this study were healthy and without symptoms of mastitis. Therefore, the factors behind these differences is not clear, however, there is a possibility that the variations could be due to differences in milk production between breasts. The variations have previously been reported in the protein composition of mammary lobes in the breast and it was suggested that the feedback inhibitor of lactation (FIL) individually regulates the protein synthesis in each mammary lobe (Murase et al., 2009). This could result in variation of protein content. Thus the possibility exists that variation observed in this study in addition to milk production might also be due to this proposed mechanism, but requires further investigation.

The variations across the day in some of the individuals were not found to be indicative of circadian variation. In addition, individual variation in protein concentrations across the day was less than variation between mothers. These results highlighted the marked variability present in milk composition between mothers, which is not associated with the maternal age, and are consistent with other studies (Clark et al., 1987; Weber et al., 2001; Bauer and Gerss, 2011).
Individual proteins have greater variability than the total protein, which supports earlier reports of greater variability in individual proteins both within and between mothers (Goldfarb et al., 1989). While we did not measure individual subunits of casein, the greater variability in the casein fraction could be related to the presence of these subunits. The individual variations in whey protein, could be due to active secretion of certain immunoglobulins by the mammary gland in some individuals (Peitersen et al., 1975), however, they were also associated with infant age. Our findings of casein and whey having large variation between mothers supports the idea that each woman has a characteristic breastmilk protein profile.

The mean casein and whey protein concentration in the present study is similar to those reported by others (Kunz and Lonnerdal, 1992; VelonÂ, 1999) during lactation. However, the mean total protein concentration in this study is slightly higher than the reported values of 10-12g/l (Saarela et al., 2005). Furthermore, the variability of less than 10% between the two methods of milk removal suggests that the method of milk expression did not alter the protein composition.

Since breast milk composition may be affected by several factors, including environmental conditions, it is important to investigate the variability in different protein fractions of breastmilk during a feed, between breasts and over the course of the day in order to determine the appropriate sampling protocol for these components. The limitation of this study includes the lack of determination of the effect of longitudinal changes on protein concentration, however this area has already been studied previously (Mitoulas et al., 2002; Kunz and Lonnerdal, 1992). Moreover, further studies are required to understand the effect of infant related factors (such as infant body weight) on milk composition.
In conclusion, no short-term changes in the concentration of whey and casein were found. This establishes that it will not be necessary to account for these short-term factors in nutritional studies. Therefore, for sampling purposes a single breastmilk sample (fore or hind milk at any time point of the day) can be used to estimate a protein concentration of skim, whey and casein fractions for a mother for that day. Although, there was no significant difference between breasts, the observation of consistent differences between breasts for three individuals indicate that milk sample from one breast cannot be reliably considered to be representative of both breasts. It is therefore recommended that samples should still be taken from both breasts of an individual mother. In addition, longitudinal changes in protein concentration should also be taken into account.

The protein concentration of skim milk, whey and casein is highly varied between mothers. Therefore, it is possible that variation in these protein concentrations between mothers may affect milk intake in infants over a 24hr period and hence be responsible for feed variation observed between exclusively breastfed infants (Kent et al., 2006). This is an area, which requires further investigation.
CHAPTER 3 Investigation of short-term variations in term breastmilk composition during repeated breast expression sessions

Sadaf Khan¹, Danielle K Prime¹, Anna R Hepworth¹, Ching T Lai¹, Naomi J Trengove¹, Peter E Hartmann¹

¹School of Chemistry and Biochemistry, The University of Western Australia, Crawley, Western Australia 6009, Australia

3.1 ABSTRACT

Background: Breastmilk composition can be affected by several factors, including short-term (weekly) variations. Investigating variations in breastmilk composition is important to accurately estimate the nutrient requirements of the infant.

Objective: To investigate short-term changes in breastmilk composition between left and right breasts and over a three-week period within first 6 months of lactation.

Method: The left and right breasts of healthy term infants’ mothers (n=23) were simultaneously expressed with an electric breast pump for 15 minutes, on three occasions, within three weeks. Milk samples (5mL) were collected from the total expression volume of each breast at each session. The macronutrient, total solids and energy content were determined using a Mid-Infrared human milk analyser. Mothers (n=17) measured their 24h milk productions and average 24h-fat contents were also determined.
Result: Over the three weekly sessions, no significant changes were found in fat, protein and lactose concentrations. On average, total solids (p=0.04) and energy (p=0.04) decreased by week three of the follow-up sessions from 14-13g/100mL and 82-76Kcal/100mL, respectively, however, these changes became insignificant when expression volume was taken into account. The macronutrient concentration was similar for left and right breasts; however, milk composition varied markedly between mothers. Furthermore, average 24h-fat content was significantly lower than the mean fat content from single expression session (p<0.01).

Conclusions: Our findings highlight that milk composition should not be assumed when determining the nutritional adequacy of a mother’s milk for her infant, and reinforces the use of average 24h-fat content of the milk to obtain the representative measures of infant energy intake.

3.2 INTRODUCTION

Wide variations have been reported in the concentration of energy yielding macronutrients (i.e., fat, protein and lactose) in breastmilk both within and between individuals (Mitoulas et al., 2002; Picciano, 2001). Breastmilk composition is affected by several factors including length of pregnancy, mother’s diet, stage of lactation, changes during a feed, and time of day (Bauer and Gerss, 2011; Mitoulas et al., 2002; Neville et al., 1984; Kent et al., 2006). Protein concentration is shown to decrease in the early stages of lactation, whereas fat content decreases and then increases as lactation progresses (Mitoulas et al., 2002). In addition, a two to three fold change has been observed in the fat content of human milk over the course of a breastfeed (Neville et al., 1984; Hall, 1979), which accounts for the high variability observed in the
energy content of human milk (Whitehead, 1995). Therefore, all levels of variation should be taken into account in order to accurately determine the nutrient content of milk.

Most previous studies investigating macronutrient variation in term human milk are based on milk samples obtained during early lactation (Lubetzky et al., 2007; Mitoulas et al., 2002; Narang et al., 2006). Only a few analysed milk samples from late lactation and to our knowledge no study has investigated the week-to-week macronutrient variation in established lactation despite the reports of acute changes in milk composition during this period (Hartmann and Prosser, 1984; Dewey et al., 1984). In addition, considerable variation in breastmilk volume was found between individual mothers and at different stages of lactation (Kent et al., 1999; Pang and Hartmann, 2007), however, little information is available regarding the influence of variations in breastmilk volume on breastmilk composition.

Since breastmilk composition is not uniform, the variability in breast milk composition could potentially influence the volume of milk consumed by breastfed infants either at each breastfeed or during the day (Neville and Robert, 1995b; Matheny et al., 1990). Therefore, increasing our understanding of the factors responsible for the variation in breastmilk composition may help explain the different feeding patterns in breastfed infants. Furthermore, without sufficient information available regarding the variation in milk composition, it is difficult to recommend the appropriate method of milk sample collection for measurement of milk constituents to estimate nutrient requirements and energy intake of infants.

This study aimed to develop a better understanding of the time-dependent variations in human milk composition during established lactation. We analysed the
macronutrients, total solids and energy in term milk samples over a three-week period within the first six months of lactation and if mother continued breastfeeding then at 6, 9 and 12 months of lactation.

3.3 MATERIAL AND METHODS

3.3.1 Subjects

Twenty-three mothers of healthy term infants participated in this study. Mothers were recruited through the West Australian branch of the Australian Breastfeeding Association, and the Child and Adolescent Community Health Nurses in the Oceanic Region.

Breast milk samples were collected on three occasions within three weeks. Some mothers also provided additional samples at 6 (n=7), 9 (n=7) or 12 (n=4) months. Participating mothers were also asked to measure their 24h milk productions. All infants (n=23) were fully breastfed on demand with a median age of 2.4 months (range 0.9 – 5.6 months) and most were male (n=17). The mean age of the participating mothers was 33±3.0 years (range 28 to 39 years) and the majority were primiparous (n=16, 70%). The mothers participating in this study have been the part of the cohort of mothers presented previously (Khan et al., 2012). All mothers provided written informed consent to participate in the study, which was approved by the Human Research Ethics Committee of The University of Western Australia.

3.3.2 Sampling

Mothers performed a 15-minute simultaneous expression of left and right breasts using two separate electric breast pumps (Symphony, Medela AG, Switzerland) on 3 occasions within 3 weeks and repeating a single session at 6, 9 and 12 months, at the
Breastfeeding Centre at King Edward Memorial Hospital, Subiaco, Western Australia (Prime et al., 2009). Milk samples were collected at each session from each breast at the beginning (pre, 1-2ml), middle (mid, 5ml) and end (post, 1-2ml) of the expression session. The total volume of milk expressed during each session was recorded. Samples were transported on ice to the laboratory where they were stored at -20°C until further analysis.

3.3.3 Mid-Infrared Analysis

A Mid-Infrared human milk analyser (HMA) (Miris AB, Uppsala, Sweden) was used to determine the macronutrient (protein, fat and lactose), total solids and energy content of the breastmilk as described previously (Casadio et al., 2010).

Mixed milk samples (5ml) collected from the total expression volume of each breast from each expression session were used to analyse the macronutrient and energy content. Prior to analysis, all samples were thawed, warmed to 40°C in a digital water bath and homogenized (1.5 seconds per 1ml of sample) using an ultrasonic processor VCX130 (Sonics & Material, Newton, Connecticut). Then a 2ml sample was injected in to the HMA for analysis using the HMA calibration mode of processed (homogenized) milk. All samples were analyzed in duplicate.

The inter-assay coefficient of variation (CV) for fat, protein, lactose, and total solid was 4.4% (SD 0.1%), 4.4% (SD 0.1%), 3.0% (SD 0.2%) and 3.1% (SD 0.3%) respectively (n=50).

3.3.4 24h Milk Production Measurements

Mothers measured their 24h milk production by test weighing their baby before and after each feed, for each breast, using electronic scales (Baby Weigh Scale, Medela AG). Mothers recorded the volume taken by the infant at each feed from each breast.
Test weighing was done at each mother’s home for a period of 24-28hrs and the measurements were later corrected to 24h, with no correction made for infant insensible water loss (Arthur et al., 1987). In addition, mothers collected and froze the small (1-2ml) milk samples before (pre) and immediately after (post) each test weighing into 5ml propylene vials (Disposable Products Pty Ltd, Adelaide, Australia).

3.3.4 Creamatocrit Analysis

The cream and fat content of all the milk samples collected during expression sessions and those collected during the 24h milk production study was measured by the creamatocrit method (Fleet and Linzell, 1964), using the Creamatocrit Plus™ device (Medela Inc. McHenry, IL, USA). Cream content of the milk is related to degree of breast fullness (Daly et al., 1993a) therefore the measurement of cream content in the samples was used to calculate the initial degree of fullness of each breast, for each expression session (Cox et al., 1996), and the amount of milk available in the breast before expression (Kent et al., 2008).

The mean 24h fat content was determined as described by Mitoulas et al. (Mitoulas et al., 2002). Briefly, pre- and post-feed milk fat contents were averaged to provide the fat content for each feed. The amount of fat taken by the infant at each feed was then determined from the volume of the feed. The sum of the amount of fat for all feeds and the total volume consumed over the study period was then used to determine an average 24h fat content (Mitoulas et al., 2002).

3.3.5 Statistical Analysis

Statistical analysis was performed using the R program, version 2.7.2 (R Development Core Team, 2009). The package nlme (Pinheiro J et al., 2009) was used for linear mixed effect modeling.
Milk intake variables (24h milk production of each breast) were compared using the paired Student’s t-test. The differences between macronutrient concentrations across the three-week period were tested using linear mixed effect models with the inclusion of week as a factor and with two possible random effects, being the effect of mother and breast within mother. Models were run for the explanatory variable (weekly interval) and for each response variable (fat, protein, lactose, total solids, energy and volume expressed). Expression volume and infant age was also tested as univariate predictors, and where significant relationship was seen they are included in the model for that nutrient. The difference between the mean fat content at a single expression session and the mean 24h fat contents were also tested using linear mixed effect models.

To test for similarities and differences in milk composition between the left and right breasts, the absolute difference between breasts at each sampling point was calculated for each of the composition variables, and then linear mixed effect models were used to test whether the differences were significantly different from zero. There were some missing values in the data (the complete 6 sample set was not obtained from seven mothers); these missing values were taken into account in analysis.

Due to the scarcity of follow up data at 6, 9 and 12 months, analysis was restricted to separately comparing each follow up time point with the initial data for that subset of individuals, using a linear mixed effects model with composition as the response and time factor as the predictor.

Univariate relationships between expression variables (volume expressed, degree of breast fullness, available milk in the breast and percentage of available milk removed) and milk composition were tested with linear mixed effects modeling.
All values are reported as mean ± standard deviation, unless otherwise stated. P values less then 0.05 were considered statistically significant.

3.4 RESULTS

3.4.1 Participants

Of the 23 mothers participating in this study, 17 measured their 24h milk production. The mean 24h milk intake of the infants was 722 ± 136 g (range 467 to 921g) and there was no significant difference between the 24h milk production from left (370 ± 109g, range 138 to 522g) and right (352 ± 121g, range 85 to 583g) breasts.

3.4.2 Milk composition over time and between breasts

The results of the biochemical analysis of macronutrient composition in milk samples over the three-week period are listed in Table 3.1. Box plots showing the distribution of data are depicted in Figure 3.1. Over the three-week period, no significant change was observed in fat, protein and lactose concentration (p=0.06, p=0.51 and p=0.86, respectively). However, significant time dependent variations were observed in total solids (p=0.04) and energy (p=0.04), with an average decrease of 0.27g and 2.4 Kcal per 100ml each week during the three-week period, respectively. Both were significantly lower at week three (p=0.04) when compared with week one and two (Table 3.1). The changes in total solids and energy became insignificant when expression volume was taken into account.

Protein concentration was significantly decreased (p<0.01) whereas fat was significantly increased (p=0.02) at the 6-month follow-up, but no change was detected after that (Table 3.2). Furthermore, lactose concentration was found to be similar during follow-up measurements (Table 3.2).
Table 3.1: Breastmilk macronutrient concentration across weekly intervals

<table>
<thead>
<tr>
<th></th>
<th>1&lt;sup&gt;st&lt;/sup&gt; week</th>
<th>2&lt;sup&gt;nd&lt;/sup&gt; week</th>
<th>3&lt;sup&gt;rd&lt;/sup&gt; week</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fat (g/100ml)</td>
<td>5.9±2.0</td>
<td>5.8±1.6</td>
<td>5.2±1.7</td>
<td>5.6±1.8</td>
</tr>
<tr>
<td>Protein (g/100ml)</td>
<td>1.0±0.2</td>
<td>1.0±0.2</td>
<td>1.0±0.2</td>
<td>1.0±0.2</td>
</tr>
<tr>
<td>Lactose (g/100ml)</td>
<td>6.3±0.3</td>
<td>6.3±0.3</td>
<td>6.2±0.4</td>
<td>6.3±0.3</td>
</tr>
<tr>
<td>Total Solid (g/100ml)</td>
<td>13.5±1.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>13.5±1.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>12.8±1.7&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>13.3±1.7</td>
</tr>
<tr>
<td>Energy (kcal/100ml)</td>
<td>82.4±17.5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>81.5±14.2&lt;sup&gt;d&lt;/sup&gt;</td>
<td>75.8±14.9&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>79.8±13.1</td>
</tr>
</tbody>
</table>

Combined values of left and right breasts for samples collected over a three-week period. Data is presented as Mean ± SD, n=23. Same letter superscripts are significantly different from each other (p<0.05).

Table 3.2: Breastmilk macronutrient concentration at follow up periods

<table>
<thead>
<tr>
<th></th>
<th>3 time points</th>
<th>6 month</th>
<th>9 month</th>
<th>12 month</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fat (g/100ml)</td>
<td>6.0±2.0</td>
<td>7.6±2.5&lt;sup&gt;*&lt;/sup&gt;</td>
<td>6.9±1.8</td>
<td>6.6±3.1</td>
</tr>
<tr>
<td>Protein (g/100ml)</td>
<td>1.0±0.2</td>
<td>0.8±0.2&lt;sup&gt;*&lt;/sup&gt;</td>
<td>0.9±0.2</td>
<td>1.1±0.1</td>
</tr>
<tr>
<td>Lactose (g/100ml)</td>
<td>6.2±0.3</td>
<td>6.2±0.2</td>
<td>6.2±0.2</td>
<td>6.1±0.5</td>
</tr>
<tr>
<td>Total Solid (g/100ml)</td>
<td>13.5±2.0</td>
<td>15.2±2.5&lt;sup&gt;*&lt;/sup&gt;</td>
<td>14.5±1.7</td>
<td>14.2±2.5</td>
</tr>
<tr>
<td>Energy (kcal/100ml)</td>
<td>82.8±17.6</td>
<td>97.0±22.6&lt;sup&gt;*&lt;/sup&gt;</td>
<td>91.4±15.6</td>
<td>89.1±25.2</td>
</tr>
</tbody>
</table>

Values are presented as Mean ± SD, n=11. Data for the samples collected on three occasions within three weeks is combined for the 11 mothers that participated in the study up to 12 months. There were 7, 7 and 4 mothers that repeated the single breast expression session and provided the samples at 6, 9 and 12 months of lactation, respectively. * Indicate significant differences (p<0.05) between the follow-up values and the three-week values for that subset of individuals.
**Figure 3.1**: Distribution of fat, protein, lactose, total solid and energy content of breastmilk across three weeks.

Weekly intervals (week 1, 2 and 3) are indicated. Box plots illustrate the median (indicated by the bold line), quartiles (box), range (error bars) and outliers (o) (n=23). Significant difference is indicated * p<0.05.

There was no significant difference between the milk from the left and right breasts of a mother in either the contents of fat (left 5.5±1.8 g/100ml; right 5.7±1.8 g/100ml; p=0.44), total solids (left 13.2±1.8 g/100ml; right 13.3±1.7 g/100ml; p=0.48) and energy (left 79.2±16 kcal/100ml; right 81.5±16 kcal/100ml; p=0.45) or concentration of protein (left 0.99±0.2 g/100ml; right 1.0±0.2 g/100ml; p=0.97) and lactose (left 6.3±0.3 g/100ml; right 6.2±0.3 g/100ml; p=0.45) across the weekly intervals (Figure 3.2). However, in five mothers consistent differences between breasts were observed in protein concentration. These mothers were found to have a consistently higher protein concentration in one breast compared to the other for all samples collected over three weeks (Figure 3.2). The mean absolute difference in the protein
concentration of milk from the left and right breast for the whole group of mothers (n=23) was 0.2±0.1 g/100ml (range 0-1 g/100ml).

**Figure 3.2:** Difference in fat, protein and lactose concentration between breasts in individual mothers.

Differences are calculated by subtracting left breast fat, protein and lactose values from the right breast. Negative values on the graph indicate that the right breast has higher concentration and a positive value indicates that the left breast has higher concentration. Each mother is represented by different symbols, with same set of symbols represents the weekly visits of each mother. The graph shows some consistent differences in left and right breast protein concentration in mothers 3(■), 8 (⊕), 12 (*), 16(□) and 22(<) in each of the three weekly sessions.

Marked variations were observed in macronutrient and energy content between individual mothers across weekly intervals. Protein content of milk samples showed a
three-fold difference ranging from 0.5-1.4g/100ml (median 1.0g/100ml, Inter quartile range [IQR] 0.9-1.1g/100ml). Lactose ranged from 4.7–7.0g/100ml (median 6.4g/100ml, IQR 6.1-6.4g/100ml). The fat content ranged from 1.3-10.5g/100ml (median 5.6 g/100ml, IQR 4.5-6.7 g/100ml). In addition, total solids and energy showed the same pattern of variation as the fat content (Figure 3.3), with total solids ranging from 9-18g/100ml (median 13g/100ml, IQR 12-14g/100ml) and energy content from 43–123kcal/100ml (median 79kcal/100ml, IQR 69-89kcal/100ml).

The fat, protein and lactose content of milk samples were not associated with either gestational age or gender of the infant.

![Figure 3.3: Pattern of variation in fat, total solids and energy in each of the 23 mothers.](image)

Values are from week 2, left breast samples and are sorted by the milk fat content.

### 3.4.3 Milk composition and expression characteristics

There was no significant difference between the expression volume for the left and right breasts (p=0.15). Over the weekly sessions, the total expression volume was
significantly increased by week three of the follow-up sessions (p=0.03), from 64 to 79ml.

Significant negative associations were found between the fat, total solids and energy content and the expression volume (p<0.01), initial degree of breast fullness (p<0.01) and amount of available milk in the breast (p<0.01), with higher fat and energy contents being associated with lower expression volumes, lower amounts of available milk and lower degrees of breast fullness. In contrast, no significant relationship was seen between protein and lactose concentration with expression volume (p=0.35 and p=0.40), degree of breast fullness (p=0.76 and p=0.08) or amount of available milk in the breast (p=0.15 and p=0.07). However, for 5 mothers with consistent between breast differences a significant relationship was observed between protein concentration and the volume of milk expressed from each breast (p=0.01), with a lower expression volume associated with higher protein concentrations. In addition, there was no significant relationship between the percentages of available milk removed and the macronutrient and energy content of milk.

3.4.4 Comparison of fat content from single expression session to the 24h fat value

The mean fat content from a single expression session, which represents the one off sample of 24h, was compared to the 24h fat content. The mean 24h fat content (4.3±0.8g/100ml, range 2.4-6.1g/100ml) was found to be significantly lower than the mean fat content from single expression session (i.e. one off sample of 24h) (p<0.01) with a mean absolute difference of 1.4±0.79g/100ml (range, 0.1-3.3g/100ml). The difference between fat content from single expression session and 24h fat content was not statistically different for left (1.4±0.9g/100ml, range 0.1-2.9g/100ml) and right (1.5±0.7g/100ml range, 0.5-3.3g/100ml) breast (Figure 3.4). The difference in the
measured fat content of milk samples collected from two different sampling protocols was inversely associated with the total expression volume (p<0.01) not to the 24h milk production.

![Graph showing absolute difference in fat value from single expression and 24h samples (g/100ml) for left and right breasts across different mothers' ID numbers.](chart)

**Figure 3.4: Absolute** difference between mean fat content from a single expression session (one off sample of 24h) and 24h fat content for left and right breast in individual mothers.

### 3.5 DISCUSSION

This study reports on changes in breastmilk composition during established lactation on three occasions within three weeks in mothers of term infants. The milk production and intake characteristics of participating mother-infant pairs were variable, but the milk production of all participants were within the normal range of these measurements (Kent et al., 2006).

Changes have been reported in breastmilk composition in relation to growth and development of the infant (Dewey and Lonnerdal, 1983; Mitoulas et al., 2002; Heinig...
et al., 1993). Protein concentration has been previously reported to decrease with the progress of lactation, rapidly during the first month and then slowly until 6 months, then remaining relatively constant (Lonnerdal, 2003; Bauer and Gerss; Saarela et al., 2005). Similarly, a rapid increase was reported in lactose concentration in the first few days of lactation, after which the lactose concentration remained constant throughout the first year of lactation (Bauer and Gerss, 2011; Saarela et al., 2005), whereas fat content decreases and then increases over the course of lactation (Mitoulas et al., 2002). Our study showed that there were no changes in protein and lactose concentrations in the short-term (across a 3 week period), which is similar to previous reports of no circadian (Mitoulas et al., 2002) and within feed variations (Hall, 1979) in these macronutrients. Although fat content is more variable (Jensen et al., 1995) and can vary within a feed and diurnally (Neville et al., 1984; Jackson et al., 1988), no significant changes (only a decreasing trend at the third week) were observed in fat content in the short-term (across a 3 week period) (Table 3.1), whereas short-term changes in total solids and energy content were related to the expressed milk volume. Furthermore, changes observed in samples collected during the follow-up visits at 6, 9 and 12 months (Table 3.2) were similar to those reported previously (Mitoulas et al., 2002; Bauer and Gerss, 2011).

These results showed that unlike early and late stages of lactation, which are characterized by considerable alterations in the composition, during established lactation, breastmilk composition is not sensitive to short-term variations (Figure 3.1). While we did not find significant differences in any of the measured macronutrients (fat, protein and lactose) between milk from left and right breasts, some mothers (n=5) did have consistent between breast differences in protein concentration (Figure 3.2).
This variation was linked to the volume of milk expressed from each breast, with each mother having one breast expressing at least 25% more milk than the other at all three weekly intervals, and this higher expression volume was associated with the lower milk protein content. This negative association between protein concentration and expression volume may be due to a dilutional effect resulting from increased milk production (Weaver et al., 1998). The individual differences and the similarity in milk composition from left and right breasts suggest that milk synthesis and secretion is controlled by both local (autocrine) and systemic factors (Neville et al., 1984; Ontsouka et al., 2003; Daly et al., 1993b).

The relationship that exists between the fat content of milk and the degree of breast fullness has previously been established (Daly et al., 1993a). It has been shown that the increase in fat content over the course of a feed is related to the degree of breast emptying i.e. the fat content increases steeply as the breast is emptied (Daly et al., 1993a; Kent et al., 2006). The present study is in agreement with these studies showing a negative association between fat content and degree of fullness (Murase et al., 2009; Saarela et al., 2005). However, for 8% of the pairs of pre- and post-milk samples in this study, the higher fat content was observed in pre-milk as compared to post-milk. This difference in the changes in fat content between pre- and post-milk might be due to the differences in either degree of fullness among mammary lobes or inconsistent emptying of various sectors of the breast (Murase et al., 2009; Kent et al., 2006).

Variation observed in the total solids and energy content of milk needs to be considered with the fact that fat is the major contributor of these components and provides up to 50% of infant energy requirements (Jensen et al., 1995). Therefore,
these variables are not independent of each other and showed the same pattern of variation as in fat (Figure 3.3). However, our results indicate that protein and lactose levels are not affected by degree of breast fullness and other expression characteristics and therefore these factors do not need to be considered when measuring these components.

The mean 24h fat content, protein and lactose concentration in the present study is similar to those reported by other investigators (Mitoulas et al., 2002; Newburg et al., 1995; Jensen et al., 1995). The large difference in mean fat content between the single expression session and over the 24h period (Figure 3.4) indicates variation in the pattern of emptying of the breast. The infants, presumably according to their appetite, empty the breast to a varying extent throughout the day. Therefore, to accurately estimate the mean fat content of a mother’s milk and infant energy intake it is important to determine the fat content from each feed from each breast over the day.

This study highlighted the variability in breastmilk composition between mothers and consequently, the average macronutrient content that they supply to their infants. Of the three major macronutrients (fat, protein and lactose), the highest variation was observed in fat content compared to protein and lactose with CVs of 34%, 19% and 5%, respectively. The limitation of the study include the small number of mothers participating in follow up sessions at different stages of lactation, and also the limited descriptive (such as absence of BMI) data of these mothers. The anthropometric data of the mothers could be helpful in explaining the inter-individual differences in the milk macronutrient concentration observed in this study.

The differences in milk composition between mothers supports the idea that each mother’s milk is individual and likely suited to the needs of her own infant. Therefore,
assuming an average concentration to determine the nutrient and energy intake of individual breastfed infants requires caution, especially when recommending earlier complementary feeding for infants based on their growth rate without knowing the actual composition of their mother’s milk. Furthermore, the variation observed in breastmilk composition between mothers may relate to the individual feeding patterns observed in mother-infant dyads, but this requires further investigation.

In conclusion, no short-term (week to week) variations were observed in the breastmilk composition during established lactation. Therefore, when sampling for nutritional studies it is not necessary to account for these weekly variations. However, we found that the mean 24h fat content was consistently lower than the mean expression fat content by approximately 14g/l. This reinforces the validity of the use of the fat content of milk samples collected over 24h to estimate the average fat content of the milk consumed by the infant during the day to obtain the representative measures of infant energy intake.
CHAPTER 4 Variation in fat, lactose and protein composition in breastmilk over 24h: Associations with infant feeding patterns

Sadaf Khan¹, Anna R Hepworth¹, Danielle K Prime¹, Ching T Lai¹, Naomi J Trengove¹, Peter E Hartmann¹

¹School of Chemistry and Biochemistry, The University of Western Australia, Crawley, Western Australia, Australia

4.1 ABSTRACT

Background: Data regarding the association between breastmilk composition and infant feeding patterns (frequency and amount of breastmilk taken) would help in understanding the regulation of food intake in breastfed infants.

Objective: This study examined the relationship between breastmilk macronutrient concentration and patterns of milk intake in breastfeeding infants over a 24h breastfeeding period.

Methods: Mothers of healthy term infants (n=15) collected pre- and post- feed breastmilk samples from each feed at each breast over a 24h period. Breastmilk samples were analyzed for fat, lactose, total protein, casein and whey protein content. The energy content for each feed was calculated.

Results: Breastfeeding patterns and milk composition varied greatly between individuals. The fat content of milk significantly differed over 24h (p= 0.01), while the concentration of lactose and protein content remained the same. The mean 24h total protein, whey and casein intake were inversely (p<0.01) whereas lactose concentration
was positively \((p=0.03)\) related to the number of breast feeds per day. No relationship was seen either between fat or energy content and feeding patterns. The average concentration of fat, lactose and total protein over the 24h period was \(43\pm12\text{g/l}, 68\pm7\text{g/l}\) and \(13\pm2\text{g/l}\), respectively.

Conclusion: The association between milk protein intake and the breastfeeding frequency suggests that the protein intake may play a role in infant appetite control.

### 4.2 INTRODUCTION

In exclusively breastfeeding mother-infant pairs, wide variations have been reported in breastfeeding frequency, volume of milk removed at each breastfeed and distribution of milk intake both during the day and night (Kent et al., 2006; Sievers et al., 2002) (Dewey and Lonnerdal, 1983). Kent et al. (Kent et al., 2006) reported a breastfeeding frequency of \(11\pm3\) times in a 24h period with a breastmilk intake of \(76\pm13\text{g}\) per feed. In addition, there was also considerable variation in daily milk intake of breastfed babies with a reported range of \(500\text{-}1300\text{ml/day}\) (Kent et al., 2006; Daly et al., 1993b; Dewey and Lonnerdal, 1983). These variations in the breastfeeding patterns suggest the existence of a self-regulatory mechanism that determines milk intake from feed to feed in breastfed infants allowing them to feed according to appetite.

Previous studies have shown that breastfed infants consume on average 67% of the available milk at each breastfeed indicating that milk intake was not restricted by maternal milk supply and was determined according to infant demand (Kent et al., 2006; Dewey and Lonnerdal, 1986). In addition, a positive relationship was identified between volume of milk consumed at a feeding and pre-prandial interval in breastfed
infants further suggesting that breastfed infants determine their feeding size, intervals and frequency to regulate their intake (Matheny et al., 1990; Neville et al., 1988).

There are large numbers of potential factors that may influence the milk intake in infants. Milk consumption by breastfed infants can be affected either by maternal (e.g. variation in milk composition and production) or infant related factors (e.g. body weight, growth related metabolic requirements, stomach storage capacities and gastric emptying rates) or a combination of both.

Data regarding the association between milk composition and infant’s level of demand are limited. There are several studies concerning the regulation of milk intake in infants, but most of them were in relation to maternal milk supply (Dewey and Lonnerdal, 1986; Daly et al., 1993b). Some studies have investigated the influence of fat content, because it is the major constituent of energy and the most variable component of breastmilk, yet these studies failed to establish the change in fat during the course of the feed as a signal for baby’s appetite control mechanism (Dorea et al., 1982; Nysenbaum and Smart, 1982).

Along with hormonal factors, gastric emptying is considered as a key regulator of appetite since rapid emptying can lead to a much earlier sensation of hunger (Hunt, 1980). Different behaviour and gastric emptying rate of each type of milk protein (casein curd and soluble whey proteins) during digestion (Khoshoo and Brown, 2002) suggest that the protein composition may either play a role in the different feeding patterns seen among breastfed infants, or have a link to infant cues for hunger. Therefore, there is a need to investigate how infant feeding patterns are influenced by the three energy-yielding macronutrients (fat, protein and lactose) with particular emphasis on the protein composition (casein and whey proteins). This study aimed to
examine the association between the variation of breastmilk macronutrient concentration over a 24h breastfeeding period and the patterns of milk intake in infants.

4.3 MATERIAL AND METHODS

4.3.1 Subjects

Breastmilk samples were obtained from fifteen mothers of healthy, term infants recruited through the Western Australian branch of the Australian Breastfeeding Association, and the Child and Adolescent Community Health Nurses in the Oceanic region. Infants were all exclusively breastfed on demand, and had a mean age of 3.2+1.6 months (range 1.0-6.0 months) and were mostly male (n=10). Mothers were between 24 and 37 years of age, (mean 31±4.0 years) and the majority of them were primiparous (n=10). All participating mothers measured their infant’s 24h milk intake and provided written informed consent to participate in the study, which was approved by the Human Research Ethics Committee of The University of Western Australia.

The number of participants was selected on the basis of two considerations, which were to include sufficient participants to cover the variation in the population, and to have data for at least 150 feeds. For these, it was considered that 15 participants was a minimum requirement based on an assumption that on average, each participant would provide the samples for 11±3 feeds (Kent et al., 2006) and this would provide the samples or data for approximately 165 feeds.
4.3.2 Milk Sampling

Milk samples 1-2ml were collected before (pre) and immediately after (post) each feed, from each breast over a 24h period by either manual breast pump or hand expression into 5ml polypropylene vials (Disposable Products Pty Ltd, Adelaide, Australia). Samples were initially stored in the mother’s home freezer for a maximum of 24h and then transported on ice to the laboratory where they were stored at -20°C until analyzed.

4.3.3 Biochemical analysis

4.3.3.1 Milk fat

The fat content of pre- and post-feed milk samples was analyzed by creamatocrit method (Fleet and Linzell, 1964) using the Creamatocrit Plus™ device (Medela Inc. McHenry, IL, USA). This method has been demonstrated to have a strong correlation with results obtained by the spectroscopic esterified fatty acid (EFA) assay (Czank et al., 2009; Mitoulas et al., 2002).

4.3.3.2 Milk lactose

The lactose content of pre- and post-feed milk sample was determined by an enzymatic spectrophotometric method (Arthur et al., 1989; Mitoulas et al., 2002). The recovery of known amount of lactose added to milk samples was 101% (standard error [SE] 0.5%, n=12). The detection limit (3*SD low concentration standard) of this assay was 0.019g/l (n=20), and the inter assay CV was 4.2% (n=60).

4.3.3.3 Milk protein

Previous analysis of changes in protein concentration in milk samples has shown no significant difference between pre & post-feed samples (Khan et al., 2012); therefore
milk protein analysis was carried out on pre-and post-feed milk samples that were pooled. Samples were defatted and then subjected to protein separation to obtain casein and whey proteins according to the method described by Kunz and Lonnerdal (Kunz and Lonnerdal, 1989; Kunz and Lonnerdal, 1990b). The protein concentration of skim (defatted milk), whey, and casein fractions of the samples was determined by the Bradford protein assay using a commercial protein reagent (Bio-Rad Laboratories, Richmond, CA, USA). Human milk protein standards were prepared by determining the concentration of an aliquot of mature breastmilk as described by Atwood and Hartmann (Atwood and Hartmann, 1992). The protein assays were carried out by the procedure described by Mitoulas et al. (Mitoulas et al., 2002). Skim milk and whey samples were diluted 1 in 30, and for casein samples a 1 in 10 dilution was used. The recovery of a known amount of the protein added to the milk samples was 99.8% (SE 1.4%, n=12). The detection limit of the assay was 0.045 g/l (n=30) and the inter-assay coefficient of variation was 6.4% (n=50).

4.3.3.4 Milk energy

The energy content for each breastfeed was calculated using the conversion factors of 9.25, 4.0, and 4.0 Kcal/g for fat, protein, and lactose, respectively (Neville and Robert, 1995b).

4.3.4 Measurement of 24h infant milk intake

Infant milk intake from each breast was determined by the test weighing procedure (Arthur et al., 1987). Mothers test weighed their infants before and after each feed, from each breast using electronic baby weigh scales (Medela AG, Switzerland; resolution 2g, accuracy ± 0.034%). Infant milk intake was calculated by subtracting the initial weight of the baby from the final weight of the baby. Test weighing was done at
each mother’s home for a period of 24-28h. The measurements were later corrected to 24h with no correction made for infant insensible water loss; therefore milk intake may be underestimated by $10\pm12\%$ (mean $\pm$ SD) (Arthur et al., 1987). Degree of breast fullness and amount of milk available in the breast was calculated from this data as described previously (Kent et al., 2006). Briefly, because there is a relationship between the fat content of the milk and the degree of fullness of the breast (Daly et al., 1993a), measuring the fat content of the samples allows the calculation of the degree of fullness of the breast before and after each breastfeeding. Degree of fullness was calculated as $1 - \text{degree of emptying}$ using the equation described by Daly et al. (Daly et al., 1993a). Minimal and maximal fat content over 24 hours correspond to degree of fullness of 1 and 0, respectively.

Since mothers were breastfeeding on demand, for this study, the 24h period was divided into four intervals of six hours and defined as morning (4:01AM to 10:01AM), day (10:01AM to 4:00 PM), evening (4:01PM to 10:00PM) and night (10:01PM to 4:00AM) (Kent et al., 2006).

4.3.5 Determination of 24h nutrient and energy intake

The protein, lactose and energy intakes of each infant were calculated from 24h milk intake and from milk composition data. In addition, the 24h fat intake was determined as described by Mitoulas et al. (Mitoulas et al., 2002). The concentration of fat for each feed was obtained by averaging the pre-and post-feed milk concentrations. The fat intake at each feed was then determined from the volume of the feed. The sum of the intake of fat for all feeds and the total volume consumed over the study period (24-48h) was then used to determine an average concentration. This average
concentration and the corrected 24h volume (Arthur et al., 1987) were then used to determine the infant total fat intake in the 24h period.

4.3.6 Statistical analysis

Statistical analysis was performed using the R program, version 2.7.2 (R Development Core Team, 2009). The package nlme (Pinheiro J et al., 2009) was used for linear mixed effect models.

The differences between macronutrient concentrations and intake over the 24h period were tested using linear mixed models with the inclusion of time of the day as a factor. Pre- and Post-feed milk samples were also compared using linear mixed models.

Univariate relationships between milk intake variables (such as frequency of breastfeeding, breastfeed volume, duration of feed, interval between feeds, 24h milk intake), and milk composition were tested with linear mixed effects model.

In all analyses different intercepts of each individual and breast within mother were used as a random effect in the model. Age of infant was tested as univariate predictor, and where significant relationship was seen it was included as covariate in the model for that nutrient (only factor for which this was significant was the whey protein concentration (p=0.048)).

Student’s paired t-test was used to compare the milk intake variables. Data was normally distributed and are presented as mean ± standard deviation, unless otherwise stated. All values are reported as mean ± standard deviation, unless otherwise stated. P values less than 0.05 were considered statistically significant.
4.4 RESULTS

4.4.1 Breastfeeding characteristics

The milk intake patterns of all 15 individual-mother infant pairs over a 24h period are presented in Figure 4.1. The mean 24h milk intake of the infants was 802 ± 161 g (range 598 to 1113 g) and was similar between the left (400±128 g) and right breasts (402±104 g). The number of breastfeeds during the day ranged from 11-19 (15±3 feeds per 24h), and the mean milk intake of the infants at a breastfeed was not significantly different from the left (64 ± 16 g) and right breasts (62 ± 18 g). Furthermore, there was no difference between left and right breasts in the time between feeds (left breast, 3.7±1.8h; right breast 3.8±1.9h) during the 24h period.
4.4.2 Milk fat

The average milk fat content was $43.2 \pm 11.8$ g/l, ranging from 28 to 57 g/l. Significant differences were observed in the fat concentration of pre- and post-feed milk samples $(32 \pm 12$ g/l, $56 \pm 17$ g/l) ($p<0.001$), respectively (Figure 4.2). Overall there were higher levels of fat in the post-milk with an average difference of 24g/l between post-and pre-milk fat content. There were significantly different baseline fat contents between
women as well as between breasts from each woman (on average). Analysis of fat content at four time periods within 24h (morning, day, evening and night) showed that fat content was significantly related to time of day (p=0.01). Compared with the morning samples fat content was higher during the day (p=0.01) and lower at night (p=0.02) with no difference between morning and evening fat concentration (Table 4.1).

The average fat content was not associated with the intervals between feeds (p= 0.15), the number of breastfeeds during the day (p=0.78), volume consumed during the feed (p=0.11), the 24h milk intake from each breast (p=0.47) or feed duration (p=0.40). The average 24h fat intake of the infant was 34.8±8.4g (range: 16-48 g) (Table 4.2) and was not related to the duration of the feed (p=0.67) or the frequency of breastfeeds (p=0.13).

4.4.3 Milk lactose

The average concentration of lactose in milk was 68±6.8 g/l and ranged from 59 to 76 g/l. No significant difference was observed in lactose concentration in pre- and post-milk samples (p=0.33) (Figure 4.2) and also between left (pre, 69.0±7.5g/l; post, 68.0±8.8g/l) and right breasts (pre, 68.0±7.7g/l; post, 67.6±7.4g/l). Analysis of lactose concentration over the four time periods within 24h (morning, day, evening and night) showed that lactose concentration was similar at all time points of the day (p=0.62) (Table 4.1). Furthermore, the average lactose concentration was not associated with the intervals between feeds (p= 0.41), the duration of the feed (p=0.21), the volume consumed during the feed (p=0.57) or the amount of available milk in the breast (p=0.21). However, there was a positive relationship between the average lactose concentration of the milk and 24h milk production from that breast (p=0.02). In
addition, a significant positive relationship was found between lactose concentration and the number of breast feedings during the day (p=0.03), with higher lactose concentration associated with more feeds per day.

The average 24h lactose intake of the infants was 56.5±14.7g (range: 29.4-78.1 g) (Table 4.2) and was not related to the duration (p=0.79) or frequency of the feeds (p=0.97).

**Figure 4.2:** Distribution of fat and lactose concentration in pre- and post- feed milk collected during breastfeeding over 24h.

Values are shown by box plots illustrating median (indicated by the bold line), quartiles (box), range (error bars) and outliers (o) (n=226 samples from 15 mothers). * Indicate significant difference (p<0.05).
### 4.4.4 Milk protein

The average protein concentrations for all mothers were found to be 13.4±2.2g/l (range 10-17g/l), 7.6±1.5g/l (range 5-9g/l) and 3.4±1.0g/l (range 2-5g/l) for skim milk, whey protein and casein, respectively.

No significant differences were observed in protein concentration of skim milk, whey protein and casein fraction over the 24h period (Khan et al., 2012). Furthermore, the interval between feeds, duration of the feed, volume consumed during the feed, breastfeed frequency and amount of available milk in the breast were not associated with average protein concentration of skim, whey protein and casein fraction.

The average 24h total protein intake of the infants was 10.9±2.62 g (range: 7.3-17.6 g) (Table 4.2) and was independent of the duration and interval between feeds. A significant negative relationship was found between the 24h total protein intake and frequency of breastfeeds (p=0.01). Thus, a higher 24h protein intake was significantly associated with fewer feeds per day.

The mean 24h whey protein and casein intake was 4.5±2.1g (range: 2.3-9.7g) and 2.1±1.1g (range:0.9-5.2g), respectively. Both the 24h whey protein and the casein intake were negatively associated with the frequency of the breastfeeds (p<0.01).

### 4.4.5 Milk energy

The mean energy content and the amount of energy delivered to the infant during the 24h period were 714±117Kcal/l (range: 540-842 Kcal/l) and 578±135Kcal/24h (range: 292-776Kcal/24h), respectively (Table 4.1).

Energy content was significantly related to time of day (p=0.01). Compared to the morning, the energy content was higher during the day (p<0.01) and lower at night (p=0.03) (Table 4.1). In addition, a significant negative relationship was found between
the energy content of the milk and degree of breast fullness (p<0.01) (Daly et al., 1993a). The energy content was not associated with intervals between feeds (p=0.25), duration of the feed (p=0.50), volume consumed during the feed (p=0.18), number of breastfeeds during the day (p=0.83) or the 24h milk intake from each breast (p=0.19). Similarly, there was no significant relationship between the total energy delivered to the infant and 24h milk intake measures.
Table 4.1: Milk composition and breastfeeding pattern over a 24h period

<table>
<thead>
<tr>
<th></th>
<th>Time of the day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Morning</td>
</tr>
<tr>
<td>Feed volume (g)</td>
<td>75±34(^{ab})</td>
</tr>
<tr>
<td>Feed duration (min)</td>
<td>12.0±4.9</td>
</tr>
<tr>
<td>Degree of Breast fullness</td>
<td></td>
</tr>
<tr>
<td>Pre-feed</td>
<td>0.63±0.23(^{a})</td>
</tr>
<tr>
<td>Post-feed</td>
<td>0.23±0.17(^{ab})</td>
</tr>
<tr>
<td>Fat (g/l)</td>
<td></td>
</tr>
<tr>
<td>Average Fat</td>
<td>40.4±11.1(^{ab})</td>
</tr>
<tr>
<td>Pre-feed</td>
<td>29.3±10.9(^{a})</td>
</tr>
<tr>
<td>Post-feed</td>
<td>52.9±16.3(^{ab})</td>
</tr>
<tr>
<td>Lactose (g/l)</td>
<td></td>
</tr>
<tr>
<td>Average lactose</td>
<td>67.7±7.6</td>
</tr>
<tr>
<td>Pre-feed</td>
<td>67.9±8.0</td>
</tr>
<tr>
<td>Post-feed</td>
<td>67.6±8.5</td>
</tr>
<tr>
<td>Total protein (g/l)</td>
<td>13.0±2.1</td>
</tr>
<tr>
<td>Whey (g/l)</td>
<td>7.5±1.6</td>
</tr>
<tr>
<td>Casein (g/l)</td>
<td>3.3±1.0</td>
</tr>
<tr>
<td>Energy (Kcal/l)</td>
<td>687±116(^{ab})</td>
</tr>
</tbody>
</table>

Data presented as Mean ± SD. Data for left and right breast for all samples within the time point of the 15 mothers is combined. The superscript letters \(^{ab}\) represents significant difference, such that time point of the day containing the same symbol were significantly different from each other (p<0.05).
Table 4.2: Macronutrient concentration and 24h infant nutrient intake from left and right breasts

<table>
<thead>
<tr>
<th>Breast</th>
<th>Macronutrient concentration (g/l)</th>
<th>Macronutrient intake (g/24h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Left</td>
<td>Right</td>
</tr>
<tr>
<td>Fat</td>
<td>43.1±12.6</td>
<td>43.3±10.9</td>
</tr>
<tr>
<td>Total Protein</td>
<td>13.4±2.1</td>
<td>13.4±2.3</td>
</tr>
<tr>
<td>Whey</td>
<td>7.7±1.5</td>
<td>7.5±1.5</td>
</tr>
<tr>
<td>Casein</td>
<td>3.4±1.0</td>
<td>3.4±1.0</td>
</tr>
<tr>
<td>Lactose</td>
<td>68.5±7.0</td>
<td>67.8±6.6</td>
</tr>
<tr>
<td>Energy (Kcal)</td>
<td>715±121</td>
<td>713±113</td>
</tr>
</tbody>
</table>

n=15. Results are mean ± SD. No statistical differences were observed.

4.5 DISCUSSION

This study has examined the association between breastfeeding patterns in exclusively breastfed infants and the macronutrient content (particularly the protein composition) of their mother’s milk. The 24h breastfeeding patterns of the participants in this study were within the normal range (Kent et al., 2006), and the mean 24h milk intake of these exclusively breastfed infants were also consistent with the previous studies for breastfed infants between 1 and 6 month of age (Kent et al., 2006; Daly et al., 1993b). However, there was considerable variation among the mother-infant pairs in the frequency of breastfeeding, breastfeed duration, interval between feeds and infant milk intake (Figure 4.1).

The present study is in agreement with previous studies showing marked variation in the milk fat content during the feed and over the course of a 24h period (Table 4.1)
(Mitoulas et al., 2002; Saarela et al., 2005; Hall, 1979; Jackson et al., 1988). Daly et al. (Daly et al., 1993a) established the relationship between fat content and extent of breast fullness and showed that approximately 70% of the variation in fat content of breastmilk was due to the changes in the volume of milk available in the breast before and after each breastfeed (Cox et al., 1996). Therefore, the two-fold increase observed in the fat content (Figure 4.2) during the feed was related to the degree of breast emptying i.e. fat content increased along with the breast emptying (Daly et al., 1993a). Similarly, higher fat content during the day compared to morning and night was due to higher degree of milk removal during the day and higher degree of fullness during morning and night (Table 4.1) which is consistent with previous reports (Kent et al., 2006; Lubetzky et al., 2007).

Our results showed no association between the changes in fat content and the milk intake in breastfed infants, which supports previous research (Dorea et al., 1982; Nysenbaum and Smart, 1982). Although fat is the most variable macronutrient of breastmilk, these results suggests that changes in fat concentration during the feed and time of the day are more likely related to the physiochemical adsorbance effect i.e. the removal of adsorbed fat globules due to the change in morphology of lactocytes upon milk ejection (Hytten, 1954a; Atwood and Hartmann, 1992) rather than to any physiological signals related to infant appetite cues. Furthermore, fat intake was independent of the frequency of breastfeeding during the day, indicating that regardless of the infant patterns of milk intake the daily fat consumption is similar between frequent and infrequent feeders (Kent, 2007). Since fat provided approximately half of the infant energy intake, infants get the same amount of energy from their mother’s milk throughout the day regardless of their feeding behaviour. In
addition, the similar patterns of variation observed in milk energy content can be attributed to the changes in fat content.

Dietary proteins have been shown to suppress the short-term food intake and have a higher satiety effect than either carbohydrates or fats in both animals and humans (Anderson and Moore, 2004). Furthermore, these effects of dietary proteins are also dependent on the protein source (Yu et al., 2009) and amount consumed as it has been shown that high protein diets are more satiating than low protein diets (Weigle et al., 2005). The milk proteins whey and casein are classified as “fast” and “slow” proteins, respectively based on their different effect on food intake in humans (Boirie et al., 1997). The digestion and absorption of whey protein amino acids is faster than casein (Boirie et al., 1997). Whey consumption also leads to higher plasma concentration of satiety hormones such as CCK, GLP-1, and peptide YY (Hall et al., 2003). In addition, the difference between the effects of whey proteins and casein on food intake may also depend on their different behaviour during digestion. Whey proteins are rapidly emptied into the small intestine as intact proteins whereas casein proteins are slowly emptied from the stomach, mainly in the form of degraded peptides. This difference in their behaviour is due to the clotting and/or precipitation of the casein (unlike the soluble whey proteins) in the acidic environment of the stomach, resulting in higher exposure of casein to gastric peptic hydrolysis (Mahe et al., 1996). However, despite the slow and fast behaviour of casein and whey proteins during digestion, Luhovyy et al. (Luhovyy et al., 2007) suggested that the combined action of whey and casein contribute to the satiety effect and the delay of the return of hunger, with whey providing early signals in contrast casein gives overlapping but late signals. In the present study, total protein intake as well as, intake of both whey
protein and casein in 24hr were found to have a significant inverse relationship with breastfeeding frequency. Overall, this is in agreement with a previous study in rats that showed that the whey protein diet is more effective in increasing the interval between meals and reducing the meal frequency compared to other protein sources (soy and gluten) (Yu et al., 2009). This suggest that milk proteins may have a role in the regulation of body weight through their satiety signals that can affect both short and long term regulation of food intake (Luhovyy et al., 2007). Formula-fed infants were shown to have higher levels of plasma insulin than breastfed infants, which can stimulate fat deposition and early development of adipocytes (Koletzko et al., 2009). It has been suggested that this change in infant’s hormonal status, which is possibly due to higher protein levels of milk formulas compared with breastmilk, may also contribute to either higher energy intake or growth velocity in formula fed infants (Axelsson et al., 1989) The protein hormones such as leptin, ghrelin and obestatin that are associated with energy intake and expenditure have also recently been found in human breastmilk (Palou and Pico, 2009; Aydin et al., 2008). While we did not assess the individual proteins, the presence of these bioactive peptides in breastmilk and the association between protein intake and metabolic regulation further strengthens the possible role of protein components in the control of appetite in developing infants. In addition, the protein intake was highly variable between infants. Therefore it is possible that this may contribute to the differences in feeding patterns as well as growth patterns of an infant, but these are areas, which require further investigation. There were no differences in the lactose concentration during the feed, between breasts and throughout the day (Figure 4.2 and Table 4.1) and this is consistent with the reports of invariant lactose concentrations in milk (Saarela et al., 2005; Mitoulas et
However, there was a relationship found between lactose concentration and breastfeeding frequency, and this may be explained by the fact that high lactose feeds are associated with rapid gastric emptying (Woolridge and Fisher, 1988). In addition, the positive association between the lactose concentration and number of breastfeeds per day is consistent with the relationship between lactose concentration and the 24h milk production of the mothers. This relationship has also been reported previously (Arthur et al., 1989; Nommsen et al., 1991) and might be due to the contribution of lactose to milk osmolarity (Nommsen et al., 1991).

The sampling protocol used in the study was similar to Hartmann et al. (Hartmann et al., 1986); we took into account all levels of variations in milk components during feeding, between breasts and over the 24h period and accurately determined the nutrient intake by the infants with minimal disturbance to infant feeding behaviour. Therefore, the mean 24h infant intake of fat, lactose and protein determined in this study (Table 4.2) agreed with the range of estimated intakes reported by others at a similar stage of lactation (Mitoulas et al., 2002; Dewey and Lonnerdal, 1983). Due to the different digestibility of casein and whey proteins, it has been suggested that low casein content in milk during early lactation may facilitate digestion, and could be an advantage for the immature digestive system of newborn infants (Lonnerdal, 2003).

The limitation of this study includes the lack of follow up at different stages of lactation, however previous studies have shown that the casein: whey ratio in human breastmilk is relatively stable during established lactation as compared to early lactation (Kunz and Lonnerdal, 1992). To our knowledge, no studies have compared the mean 24h intake of casein and whey proteins in breastfed infants. However, to make dietary recommendations regarding these proteins requires further studies,
preferably longitudinal studies with specific functional outcomes (such as the relation of whey protein and casein to growth patterns in infants) are required.

In summary, the association between milk proteins (whey protein and casein) and frequency of breastmilk intake suggests that protein composition may have a role in regulation of food intake and necessitates studying both the short and long-term effect of protein on infant growth. Apart from the dietary constituents of a feed, several interacting factors are involved in the regulation of appetite and energy intake, including gastrointestinal and hormonal factors. Therefore, to completely understand the association between feeding patterns in breastfed infants and appetite control physiology it is important to consider all of these aspects, in particular gastric emptying rates and infant’s stomach storage capacities. The complete understanding of early programming of infant appetite needs to be explored as infant feeding practices have the potential to contribute to the development of infant and child obesity.
CHAPTER 5  Ultrasonic assessment of gastric volume and emptying in term breastfed infants

Sadaf Khan\textsuperscript{1}, Anna R Hepworth\textsuperscript{1}, Naomi J Trengove\textsuperscript{1}, Peter E Hartmann\textsuperscript{1}, Donna T Geddes\textsuperscript{1}

\textsuperscript{1}School of Chemistry and Biochemistry, The University of Western Australia, Crawley, Western Australia, Australia

5.1 ABSTRACT

Background: Ultrasound is a non-invasive imaging method used to measure the infant stomach. Limited data is available on the influence of breastmilk volume and composition, on gastric emptying in term infants.

Objectives: To validate and assess the reliability of ultrasonic measurement of stomach volume in term infants and to determine the relationship between gastric emptying (GE) and the volume and macronutrient content of a breastfeed.

Methods: Transverse and sagittal ultrasound scans of the infant stomach were performed before and after one feed (n=18) and at 10-20 minute intervals (n=9) until the next feed to measure the stomach volume. The measurements were performed in duplicate and all ultrasound images were examined to assess and grade the post-feed echogenicity of stomach contents, curd density and curd volume. Gastric emptying was determined by calculating the proportion of the volume of the feed remaining in the stomach, which was correspondent to the decrease in stomach volume. Breastmilk
samples (~5ml) were collected and analyzed for fat, lactose, total protein, casein and whey protein content and the energy content was calculated. Milk intake was measured by the test-weigh method.

Results: Milk intake (71 ± 38ml) was positively correlated with stomach volume as measured by ultrasound (p<0.001, R²=0.90). Duplicate measurements were highly consistent (ICC>0.95) and unbiased. The stomach contents displayed different patterns and echogenicity immediately after the feed and were significantly related to the casein concentration (p=0.02) and the ratio of whey to casein (p=0.01). There was no significant relationship between the proportion of feed remaining and the macronutrient and energy content of the feed. However, gastric emptying was inversely related to the volume of milk taken during the feed (p=0.04), with higher milk intake associated with slower emptying.

Conclusion: Ultrasound is a reliable method to monitor the gastric emptying in term infants and is able to describe intra-gastric curding. The volume not the macronutrient content of the feed appears to play a role in the control of gastric emptying in term infants and requires further investigation.

5.2 INTRODUCTION

Normal growth and development of infants is critically dependent on the efficient delivery and absorption of nutrients derived from the feed (Woodley and Mousa, 2008). Gastric emptying is involved in the release of nutrients from the stomach to small intestine for further digestion and absorption; therefore this process has a metabolic impact and has also been implicated in the control of feeding behaviour in human adults and animals (Lorenz, 1985; Hellstrom et al., 2006).
Gastric emptying rate (GER) is influenced by characteristics of the milk, including nutrient composition, nutrient state, osmolality and volume (Hunt and Stubbs, 1975; Siegel et al., 1985; Salvia et al., 2001; Lin et al., 1992). For example, feeds with either high energy density or osmolality may delay gastric emptying (Siegel et al., 1984). However, most of the studies that have assessed the effect of feed characteristics on gastric emptying are based on formula milk (derived from cow’s milk), which differs considerably from the breastmilk in nutritional composition and curding properties (Lonnerdal and Atkinson, 1995). Human milk composition varies both between and within women (Pang and Hartmann, 2007) and marked variations have been reported in feeding patterns and total milk intake within and between mother-infant pairs throughout the day (Kent et al., 2006). Given these differences it is assumed that the relationships of different nutrients to gastric emptying as shown in case of formulas are also valid for human milk, which requires investigation.

In addition, previous studies have suggested that varying the protein composition (i.e. two major milk proteins, whey and casein) of the feed influences GER (Fried et al., 1992; Billeaud et al., 1990; Tolia et al., 1992). When infants ingest the milk, casein precipitates in the stomach in response to the acidic gastric pH. During this process, the milk components are separated into successive layers of semi solid and liquid phases known as curd and whey. Casein and lipids are trapped in the curd, which is digested slowly. The fluid portion (whey) consists of other milk components such as water; whey proteins, lactose, oligosaccharides and minerals and passes rapidly through the stomach into small intestine (Mahe et al., 1996; Miyazaki et al., 2009; Longenbach and Heinrichs, 1998). The physiological formation of curd and the differential rate of emptying of the two phases (semi solid and liquid phase) of the
ingested milk have been shown to have clinical significance and are important for efficient digestion and absorption of nutrients in infants (Khoshoo and Brown, 2002; Khoshoo et al., 1996). However, previous studies have not reported intra-gastric curding or factors that might influence gastric curding in infants.

There is very limited data available regarding the importance of gastric volume in the control of gastric emptying in infants. It has been shown in pre-term infants that altering the feed volume and osmolality simultaneously influences gastric emptying, with increased feed volume and decreased osmolality increasing gastric emptying (Ramirez et al., 2006). Moreover, in breastfed term infants the volume of breastmilk intake has not been investigated as a factor contributing to gastric emptying.

Ultrasound imaging is a non-invasive method that can be used to measure the infant stomach and visualize the gastrointestinal organs and their contents. The established ultrasound imaging methods to evaluate gastric emptying include the measurement of antral cross section area of the stomach (Newell et al., 1993) and direct measurement of stomach volume (Lambrecht et al., 1988). The direct measurement technique involves scanning the stomach in its entirety, enabling assessment of the gastric contents (although this has not been described) whereas images of the antral cross sectional area do not provide this information. Although the previous ultrasound studies have provided the foundation to measure the stomach volume, validation and the determination of reliability and repeatability of the measurements is required to ensure the accuracy of this tool. In addition, the ultrasonic appearance of breastmilk curding has not been investigated.

This study had three objectives: The first, to validate the ultrasonic measurement of infant stomach volume to monitor gastric emptying and determine the reliability and
repeatability of these measurements. The second, to assess the visible differences in milk curding and curd volume in the stomach recorded by ultrasound images in relation to milk composition and milk volume. The third objective was to investigate the relationship between gastric emptying and both the macronutrient content and volume of a breastfeed (feeding characteristics) in term infants.

5.3 MATERIAL AND METHODS

5.3.1 Participants

Twenty-seven lactating mothers and their healthy term infants were recruited through either the Australian Breastfeeding Association or community health centres. The mother-infant pairs were divided into two groups. The first group of mother-infant dyads (n=18) participated in study A for validation and assessment of reliability of ultrasound measurements of stomach volume. The infants participating in study A were 5 and 11 weeks of age (mean ± SD, 7±2 weeks, n=3 age unknown). Some of these infants were scanned for both a breastfeed (n=12) and bottle-feed [(expressed breastmilk (EBM)] (n=12) or a combination of both feeds (mixed feed) (n=4). The second group (n=9) participated in study B, investigating the relationship between gastric emptying and feeding characteristics, and the infants were 4-20 weeks of age (mean ± SD, 10±6weeks). The weight of the infants participating in study B was recorded and they were all scanned for a breastfeed except one infant, which was fed from both breast and a bottle containing EBM. Ultrasound imaging of gastric contents was assessed in both groups. The infants (n=27) had a mean age of 8±4 weeks (range, 4-20 weeks) and 56% were male. The infants were scanned at the Breastfeeding Centre of Western Australia. The Human Research Ethics Committee of The University
of Western Australia approved the study, and all mothers gave written consent to participate in the research.

5.3.2 Sampling
Milk samples (~5ml) were collected into a 5ml polypropylene vial from the participating mothers. In cases of expressed breastmilk and mixed feeds, an aliquot was obtained from the total expressed milk. For a breastfeed, samples were collected by hand expression before and after the feed from one breast and were pooled for analysis.

Milk samples were transported on ice to the laboratory where they were kept at -20°C until further analysis.

5.3.3 Biochemical Analysis

5.3.3.1 Milk Fat
The fat content of milk samples were measured by a spectroscopic esterified fatty acid method (EFA) (Mitoulas et al., 2002; Stern and Shapiro, 1953). Briefly 2.5 µl aliquots of milk samples (warmed to 25°C) and standards (Triolein; Sigma-Aldrich, NSW, Australia) were dispensed into 1.5ml propylene tubes containing 600µl redistilled ethanol and mixed thoroughly. 2M hydroxylamine hydrochloride (100µl) and 3.5M NaOH (100µl) were then added to each tube and after mixing tubes were left to stand at room temperature for 20min, then acidified with 4M HCl and colour production was achieved by the addition of FeCl₃-TCA solution (7.5g TCA in 0.37M FeCl₃-0.1M HCl). The tube contents were mixed and 250µl from each tube was pipetted into 96-well microtitre plate in duplicate and the plates were read at 540nm using a plate
spectrophotometer (Titertek Multiscan MCC/340; Flow laboratories, McLean, VA, USA).

The detection limit of this assay was 3g/l and the inter assay coefficient of variance (CV) was 7% (n=20).

5.3.3.2 Milk Lactose

The lactose content of milk samples was determined by an enzymatic spectroscopic method (Mitoulas et al., 2002; Arthur et al., 1989). Briefly defatted milk samples and lactose standard were diluted 1 in 150 with distilled deionized water and were pipetted into duplicate wells on a flat bottom 96-well microtiter plate. Reagent 1 (8U β-galactosidase/ml in 0.1M phosphate buffer, pH 7.2) was then added to each well and the plate was mixed and incubated for 1hr at 37°C. Following this, reagent 2 (9.6U glucose oxidase/ml, 2.5U peroxidase/ml, 500ug 2,2-azino-di-(3-ethyl-benzthiazolinsulfonate)-6-sulfonate/ml in 0.1M potassium phosphate buffer, pH 7.2) was added to each well. Absorbance was determined at 405nm using a plate spectrophotometer at 5 min intervals for approximately 45min until a peak absorbance was reached.

The recovery of a known amount of lactose added to milk samples was 101% (standard error [SE] 0.5%) (n=12). The detection limit of the assay was 0.02g/l (n=20) and the inter assay CV was 4.2% (n=60).

5.3.3.3 Milk Protein

Milk samples were thawed, defatted and subjected to protein separation to obtain casein and whey proteins as previously described (Kunz and Lonnerdal, 1989; Kunz and Lonnerdal, 1990b). The protein concentration of skim (defatted milk), whey, and casein fractions of the samples was then determined by the Bradford protein assay
using a commercial protein reagent (Bio-Rad Laboratories, Richmond, CA, USA). Human milk protein standards were prepared by determining the concentration of an aliquot of mature breastmilk by Kjeldhal method to calibrate the measurements (Atwood and Hartmann, 1992). The protein assays were carried out by the procedure described by Mitoulas et al. (Mitoulas et al., 2002). Briefly, skim milk and whey samples were diluted 1 in 30 and casein samples 1 in 10 with double deionised water and pipetted (5 ml) in duplicate, with standards, onto a 96-well microtitre plate. The Bio-Rad protein assay reagent (diluted 1 in 5 with distilled deionised water) was added to each well, the plate was mixed and absorbance was then measured at 620 nm using a plate spectrophotometer. The recovery of a known amount of the protein added to the milk samples was 99.8% (SE 1.4%, n=12). The detection limit of the assay was 0.045 g/l (n=30) and the inter-assay CV was 6.4% (n=50).

5.3.3.4 Milk Energy
The energy content of the milk samples were calculated using the conversion factors of 9.0, 4.0, and 4.0 Kcal/g for fat, protein, and lactose, respectively (Neville and Robert, 1995b).

5.3.4 Ultrasound Examination
The infants’ stomach were scanned using the Toshiba SSA-770A/80,Apio 80 (Tokyo, Japan) ultrasound machine with a PVT-674BT (6MHz) transducer and Parker ultrasonic gel (Fairfield, NJ, USA). Scans were performed in the supine position with the head slightly raised. An experienced ultrasonographer has performed the scans and all images were recorded for later analysis.
5.3.4.1 Gastric volume measurement

Ultrasound scans of the infants stomach was performed before and after the feed to measure the pre- and post-feed stomach volumes. The stomach was scanned in its entirety and the maximal sagittal, transverse, and anterior-posterior axes were determined for the calculation of stomach volume.

The longitudinal, anterioposterior and transverse diameters of the stomach were later measured from the archived ultrasound images using the Screen Callipers (Inconico, V4). All measurements were performed in duplicate.

The gastric volume (ml) was then calculated from the measured maximum stomach length, anterioposterior and transverse diameters using the formula for an ellipsoidal body.

\[
\text{Stomach volume (ml)} = \text{longitudinal diameter} \times \text{anterioposterior diameter} \times \text{transverse diameter} \times 0.52
\]

5.3.4.2 Gastric emptying assessment

Gastric emptying was recorded by measuring the infant stomach volume prior to the feed, immediately after the feed (0 min) and then at regular intervals of 10-20mins until the baby cued for next feed.

Stomach volumes were standardized as the post stomach volume obtained just after the feed (0 min) was considered 100% (\(S_0\)). The proportion of feed remaining in the stomach was then calculated, which was correspondent to the decrease in stomach volume, by transforming all the following post stomach volume determinations (\(S_n\)) as a percentage decrease in \(S_0\) as follows:

\[
\text{Proportion of feed remaining in the stomach (\%) } = \left( \frac{S_n}{S_0} \right) \times 100.
\]
5.3.4.3 Ultrasound imaging of gastric content

All recorded ultrasound images were examined to assess the echogenicity of the milk in the infant stomach and to quantify the curd density and volume. The echogenicity or pattern of the gastric feed content was graded as higher (hyperechoic compared to adjacent spleen), lower (hypoechoic compared to spleen), mixed (intermediate echogenicity) and snow storm (Figure 5.1).

The curd formed in the stomach was graded by the changes observed in its echogenicity at different time intervals. The splenic echogenicity was taken as a standard to compare the degree of echogenicity (or density) of the curd. The curd density was then categorized as follows (Figure 5.2):

High: when curd was more echogenic (brighter) than spleen
Low: curd was less echogenic (darker) than the spleen, and
Same: curd echogenicity (brightness) was the same as spleen

The extent of curding (curd volume) was also determined from the same ultrasound images by calculating the proportion of the stomach filled with the visible curd and were categorised as High (when more than half of the stomach was filled) and Low (less than half of the stomach was filled with curd).
Figure 5.1: Ultrasound images of the infants’ stomach immediately after the feed.
Post-feed images showing different patterns of appearance of feed contents A) Highly Echogenic B) Low echogenicity C) Snow storm pattern D) Mixed echogenicity.
Figure 5.2: Comparison of observed post-feed curd density in the stomach with spleen. (A) High curd density, curd brighter than spleen (B) Same curd density, same brightness of curd and spleen.

5.3.5 Determination of Milk intake

The amount of milk consumed by the infant during the monitored feed was determined by test weighing the baby before and after feeding using an electronic balance (Medela Electronic Baby-Weigh Scales, Medela AG, Switzerland). The milk intake was calculated by subtracting the initial weight of the infant from the final weight of the infant (Arthur et al., 1987).

5.3.6 Statistical Analysis

Statistical analysis was carried out using R 2.7.2 for Mac OSX (R Development Core Team, 2009). The packages nlme (Pinheiro J et al., 2009), irr (Gamer et al., 2007), multcomp (Hothorn et al., 2008) and lattice (Sarkar D, 2008) were used for linear mixed modelling, intraclass correlations, multiple comparison of mean and graphical exploration of data, respectively.
Descriptive analysis is reported as mean ± SD for normally distributed data and as median (range) otherwise. P values less then 0.05 were considered statistically significant.

5.3.6.1 Reliability of ultrasound measurements

The replicate measurements were compared graphically using Bland and Altman plots (Bland and Altman, 2002) and analytically using intraclass correlation coefficient (ICC) (McGraw and Wong, 1996; Shrout and Fleiss, 1979). Bland-Altman plots were used to investigate whether there was any difference between measurements that related to the magnitude of the measurement.

To determine the reliability, measurement bias was assessed by calculating the mean and limits of agreement (Mean±2*SD) of the differences between replicates (rep2-rep1). The measurements were considered to be biased if the interval between the limits of agreement did not contain zero. The ICC was calculated to assess the degree of reliability (Nunnally and Bernstein, 1994), and was calculated for agreement (absolute difference between replicates) and consistency (different pattern of differences between replicates). Confidence intervals (CI) (95%) were also calculated. Consistency and agreement scores were considered to be significantly different if the CIs did not overlap. The reliability of the measures was judged using the guidelines presented in Nunnally and Bernstein (Nunnally and Bernstein, 1994), with a minimum ICC of 0.70 being required as a measure of acceptable agreement or consistency for use in the study and minimum of 0.95 for measures to be transferred to a clinical setting.
5.3.6.2 Validation of gastric volume measurements

Ultrasound measurements of stomach volume and change in stomach volume (post-feed stomach volume – pre-feed stomach volume) were validated against the known feed volume measured by test weighing technique. Linear mixed models with no intercept, and random effects of individual intercepts were tested against ordinary least squares regression models using analysis of variance, and the appropriate model selected.

5.3.6.3 Comparison of ultrasound image based quantification and feeding characteristics

To compare the echogenicity, curd density and curd volume to the milk composition and intake, the ultrasound images of the infant stomach taken just after the feed was used. There were some missing values in the data (the composition data was not available for 6 infants participating in the gastric volume validation study); which were not included in the analysis. In two infants no visible curd was seen at the immediate post-feed visualization, these infants were not included in the curd density and volume analysis. In this data set, the observed curd volume was either high or low and there were three levels of curd density high, low and same. In addition, the echogenicity has four grades (high, low, snowstorm and mixed).

To test the relationship between the milk composition and intake, with the echogenicity and curd density measures, least squares regression was used. A multiple comparison of means (Tukey’s all pair comparison) was also used to test the means of each echogenicity and curd density group against each of the others when significant results were seen in post-hoc tests.
Logistic regression was used to determine whether the possibility of occurrence of the two categories in curd volume, which was high and low, differs according to composition of the milk sample and volume of the feed. In this analysis curd volume was treated, as the response and milk composition and intake were the predictors.

Fisher’s exact test was used to test the effect of feed type (breast or bottle-feed) on the observed stomach conditions (echogenicity, curd density and curd volume).

5.3.6.4 Relationship between gastric emptying and milk intake and composition

The proportion of feed remaining in the stomach was considered as an estimate of gastric emptying and used to determine the effect of feed characteristics (milk composition and intake) on gastric emptying.

Linear mixed models were used to determine whether there was any relationship between proportion of feed remaining in the stomach and any of the milk composition variables (fat, total protein, whey, casein, lactose and energy) and between milk intakes. For each model, the random effects grouping were the individuals, and time was included as a covariate. Univariate relationships between the proportion of feed remaining in the stomach and infant weight, age and gender were also tested with linear mixed effects model. In the model that tests the relationship between the proportion of feed remaining and milk intake, weight of the infant was also included as covariate.

To determine whether there was a relationship between the feed proportion remaining in the stomach and the qualitative properties of stomach contents (echogenicity, curd density and volume), a linear mixed effects model was created for each property, with proportion of feed remaining as the response, a categorical variable for the property and the random effects as above.
5.4 RESULTS

5.4.1 Participant characteristics

The mean age and parity of the participating mothers (n=21) were 35±5 (range: 28-44) years and 2±1 (range: 1-6) children (for n=6 mothers, age and parity unknown). All mothers were predominantly breastfeeding their infants and had no concerns about their lactation. The weight of the infants (n=9, participating in Study B) was 3782-7268g (mean ± SD, 4972±1002g). The infants were healthy and growing appropriately for their age according to World Health Organization’s growth charts for breast-fed infants (World Health Organization).

5.4.2 Reliability of ultrasound measurements

There was a high agreement between the first and second replicates of the rater and no measurement bias was detected. The pre- and post-feed measurements were independent of each other as no increase in the size of the discrepancy was found with the increase in measurement (Figure 5.3). There were only two discrepancies (one each in anterioposterior and longitudinal) diameter measurements that were ~4mm. Similarly; in calculated stomach volumes only two discrepancies of around 5ml were found, and they were for the milk intakes of 145 and 106ml (Figure 5.3). Mean and standard deviations of the differences between the replicate measurements are presented in Table 5.1. All measurements had good ICC agreement and consistency scores and were above the clinically useable threshold of 0.95 (Table 5.1).
Figure 5.3: Bland-Altman plots showing the difference between the first and second replicates of the rater for each of the ultrasound measurement.

AP= Anterioposterior diameter, LG= Longitudinal diameter, TR=transverse diameter and SV= Stomach volume
Table 5.1: Mean, SD and limits of agreement of the difference between replicates (rep2-rep1) and ICC agreement and consistency scores by rater

<table>
<thead>
<tr>
<th></th>
<th>Limits of agreements</th>
<th>ICC agreement (95%CI)</th>
<th>ICC consistency (95%CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>Lower</td>
</tr>
<tr>
<td>Anterioposterior (mm)</td>
<td>0.10</td>
<td>0.95</td>
<td>-1.8</td>
</tr>
<tr>
<td>Longitudinal (mm)</td>
<td>-0.01</td>
<td>0.68</td>
<td>-1.3</td>
</tr>
<tr>
<td>Transverse (mm)</td>
<td>-0.05</td>
<td>1.14</td>
<td>-2.3</td>
</tr>
<tr>
<td>Volume (ml)</td>
<td>0.22</td>
<td>1.89</td>
<td>-3.5</td>
</tr>
</tbody>
</table>

5.4.3 Gastric volume and Milk intake

In 54% of the pre-feed measurements, the stomach was found to be completely empty with no visible residual in the stomach. The median pre-feed volume was 10ml (range, 4-156ml).

There was a significant relationship between the stomach volume measured immediately after a feed by ultrasound imaging and the milk intake volumes determined by test weights (p<0.001, R²=0.90). However, the relationship became stronger when the estimate of the volume taken during the feed was determined by the change in stomach volume (pre-feed stomach volume-post feed stomach volume) (p<0.001, R²=0.99) (Figure 5.4).
The mean milk intake determined by test weighing was $71\pm38$ml (range: 20-145ml) and the post-stomach volume measured by ultrasound (including residual) was $83\pm49$ml (range: 23-212ml). The mean corrected stomach volume (post-feed stomach volume – pre-feed stomach volume) was $71\pm37$ml (range: 22-142ml).

![Figure 5.4: Milk intake of the infant in relation to the stomach volume measured by ultrasound. The grey dotted lines indicate the 10ml difference and the black line indicates the similarity between the determined volumes from two different methods.](image)

5.4.4 Post-feed ultrasound imaging of gastric contents

The stomach contents displayed different patterns and echogenicity immediately after the feed (0 min) between infants. The gastric contents were visualized as either a predominantly echogenic (white) image corresponding to curdled portion of the feed (graded as high), a mainly anechoic (black) image corresponding to the fluid portion of the feed and some intermediate echogenicity curd surrounded by the echogenic stomach wall (graded as mixed), several very small curds visible as echogenic flecks.
floating in the stomach (described as snow storm) or a clot of low echogenicity curd larger than floating curds and an anechoic image (graded as low) (Figure 5.1).

5.4.5 Ultrasound image based quantification and feeding characteristics

Box plot analysis of the association between image based classification of the stomach echogenicity, curd density and curd volume observed at the end of the feed to the macronutrient content of the milk samples are presented in Figure 5.5 and 5.6.

There was no significant relationship between the stomach echogenicity and the fat content (3.4±17g/l), lactose (80±10g/l), total protein (13±3g/l) and whey (8±2g/l) concentration of the milk samples. However, the echogenicity of the stomach was significantly related to the casein concentration (p=0.003) and the ratio of whey to casein (p=0.01). There was a significant difference between the mean total casein (3±2g/l) concentration and whey to casein ratio for the four grades of stomach echogenicity. The average casein concentration in mixed grade (4.4±1.3 g/l, n=11) was significantly higher than the snowstorm (2.5±1.1g/l, n=9; p=0.01) and high (1.8±1.2g/l, n=4; p=0.01) grades, while there was no difference between the mixed and low (3.0±1.1g/l, n=3) grade (p=0.33). In addition, no significant difference was found in casein concentration when comparisons were made among other grades of stomach echogenicity, which were between snowstorm and high, snowstorm and low; and low and high grades. In contrast, the whey to casein ratio was lowest in the mixed group (2.0±1.1) compared to snow (3.4±1.2), high (5.0±2.7) and low (2.8±1.5), however only the difference between high and mixed grade was significant (p=0.01). There was no significant relationship between the three levels of curd density (high, low and same) and the fat, lactose, total protein, whey and casein concentration of the
milk samples. Similarly, no association was found between the curd volume and fat, lactose and individual proteins (whey and casein) content of the milk.

In addition, volume of milk taken during the feed was not significantly different among mixed (69±39ml,n=20), high (50±30,n=4ml), low (115±66ml,n=3), and snowstorm (95±38ml,n=10) grades of echogenicity (Figure 5.7). Similarly, no relationship was found between milk intake and post-feed curd density and volume (Figure 5.7). Moreover, no effect of the type of the feed (i.e. breastfeed or EBM bottle feed) was observed on the echogenicity, density and volume of the curd.

**Figure 5.5:** Association between ultrasound image based classification of stomach echogenicity observed at the end of the feed to the macronutrient content of the milk samples.

Box plots illustrate the 95% confidence interval of the mean (notches), median (middle bold line), quartiles (box ends), range (error bars) and outliers (o) (n=27). * Indicate significant difference (p<0.05).
Figure 5.6: Association between ultrasound image based classification of post-feed Curd density (A) and Curd volume (B) to the macronutrient content of the samples. Box plots illustrate the 95% confidence interval of the mean (notches), median (middle bold line), quartiles (box ends), range (error bars) and outliers (o) (n=27). No significant difference was found.
**Figure 5.7**: Box plot analysis of the association between stomach echogenicity, curd density and curd volume grading to the milk volume taken during the feed. No significant difference was found (n=27).

### 5.4.6 Gastric Emptying (GE)

The pattern of emptying in individual infants is shown in Figure 5.8. There was a strong curvilinear (quadratic) relationship between the percentage of feed remaining in the stomach and the time since feed (p<0.001).

GE was significantly related to the volume of milk taken during the feed (p=0.04), with higher milk intake being associated with slower emptying. However, no significant relationship was observed between the GE and fat (35±11g/l, p=0.20), lactose (73±3g/l, p=0.33) and energy (544±56kcal/l, p=0.73) content of the feed. In addition, there was no significant relationship between the GE and total protein (12±3 g/l, p=0.40), whey (8±3g/l, p=0.38) and casein (2.5±1.2g/l, p=0.24) concentration of the
milk samples. The GE was not associated with the weight and either age or the gender of the infant.

The changes observed in the curd density were related to the proportion of the feed remaining in the stomach during emptying. Significantly high curd density was observed when most of the feed emptied from the stomach (p=0.04), whereas no visible curd was seen when higher proportions of feed were remaining in the stomach (p=0.03). Similarly, the amount of the visible curd (curd volume) was also related to the average proportion of feed remaining in the stomach, with high curd volume being seen with lower proportions of the remaining feed contents (i.e. later in the emptying) and none or low curd volume being seen when the proportion of the remaining feed was higher (i.e. at the start of the emptying process).

In addition, individual patterns were observed with respect to the curd density and volume during emptying between infants. In 2 out of 9 infants investigated, no visible curd was seen in the stomach immediately after the feed with the volumes of 90 and 140ml whereas in the remaining infants curd was evident immediately.
Figure 5.8: Pattern of gastric emptying in individual infants. The dotted lines represent the half of the feed (50%) remaining in the stomach.

**5.5 DISCUSSION**

In this study we validated and tested the accuracy of the ultrasound imaging method to measure infant stomach volume for the investigation of the influence of breastmilk on gastric emptying in term infants. This study has shown that ultrasound is a consistent and reliable method for measurement of infant gastric volume and to monitor gastric emptying in relation to breastmilk composition.

Apart from ultrasound a number of other non-invasive methods have been used to assess gastric emptying including scintigraphy, the stable isotope breath test and magnetic resonance imaging (MRI) (Szarka and Camilleri, 2009). However these methods have certain technical limitations and are difficult to perform, thereby
restricting their use in infants for both clinical and research purposes. For example, scintigraphy, which is considered as gold standard for monitoring the GE in adults in both clinical and research settings, is not feasible to use in infants because it involves exposure to ionising radiation (Gomes et al., 2003). The stable isotope breath test is a non-imaging method and MRI requires expensive equipment and sedation of the neonates making both of these methods unsuitable for research (Szarka and Camilleri, 2009; Van Den Driessche et al., 1999). In contrast, potential biological effects from ultrasound are much less hazardous than X-radiation. Ultrasound also has the added advantages of being non-invasive, portable, cost effective, widely available and relatively easy to perform making the technique preferable to other methods, especially in infants (Gomes et al., 2003; Lambrecht et al., 1988). These attributes were key to choosing this method to investigate gastric emptying in term breastfed infants.

Ultrasound imaging has been used previously to investigate gastric emptying in preterm infants and children. Most often serial measurements of gastric antral cross section area (ACSA) were employed (Ewer et al., 1994; McClure and Newell, 1996). We however evaluated gastric emptying, by direct measurement of stomach volume at regular intervals as this technique unlike ACSA allows the assessment of entire stomach (Lambrecht et al., 1988). Several longitudinal and transverse scans, which were perpendicular to the longitudinal axis, were taken to determine the longest and widest transverse axis of the stomach and the stomach volume has been calculated using the formula of an ellipsoid body.

In adults and children the ultrasound studies of GE have been validated by comparison to barium and scintigraphy studies (Darwiche et al., 1999; Gomes et al., 2003). The use of these radiological and radionuclide methods for validation purpose is not feasible or
ethical in infants. Furthermore, in pre-term infants ultrasound measurements of gastric antral volume was validated by measuring the ACSA after administration of 25, 50, 75 and 100% of feed volume and a linear correlation between the ACSA and the volume of ingested or administered liquid was demonstrated (Newell et al., 1993). We chose to determine the validity of the ultrasound method by comparison with the test weighing method, which is used to determine the milk intake in breastfed infant (Arthur et al., 1987). A significant linear relationship between the gastric volume and the volume of ingested milk was found confirming the accuracy of ultrasound in determining gastric volume despite no correction for stomach residuals. Indeed the relationship became stronger when the estimate of milk intake was determined by the change in stomach volume and there was only one observation where stomach volume was underestimated by ~10ml (Figure 5.4). This demonstrates the validity of measuring gastric volume by ultrasound imaging. Furthermore, in order to assess the reproducibility all the measurements of longitudinal, anterioposterior and transverse diameter of the stomach were performed in duplicate. The reliability of these measurements was found to be well within the limits of ICCs consistency and agreement scores (Nunnally and Bernstein, 1994) with only two discrepancies of up to 4mm observed, reflecting the accuracy of the technique (Figure 5.3).

Since most of the previous gastric emptying studies were carried out in hospital settings in pre-term and sick infants receiving enteral feeds, it was not difficult to control the feed volume, interval between the gastric measurements and duration of the examination in these infants (Ramirez et al., 2006; Siegel et al., 1984). In contrast our study, was performed in healthy demand breastfed infants without disturbing the normal feeding pattern, which limits the regularity of scan intervals and consistency of
feed duration. In our study some infants exhibited feeding cues within 30 minutes of their last feed while others cued well after an hour resulting in the number of time points in our study to be somewhat limited and uneven. Concerning the measurements, the calculations were simplified by expressing the GER in terms of proportion of feed remaining in the stomach which avoided the individual variations, rather than using the complicated logarithmic evaluation of the gastric volume to determine the statistically defined half emptying time \( (t_{1/2}) \) (Bateman, 1982b; Lambrecht et al., 1988; Ewer et al., 1994).

However, despite the limited serial measurements the curvilinear (neither exponential nor linear) pattern of emptying observed in our study (Figure 5.8) with more rapid initial phase followed by a slower one, was similar to the recognized patterns of gastric emptying reported by others in preterm infants (Carlos et al., 1997; Cavell, 1979; Ewer et al., 1994). In addition, there was wide variation between infants in emptying of 50% of the feed from the stomach; the time approximately ranges from 15-49 minutes (mean 30±12.5 min) (Figure 5.8), which is similar to the previously reported mean range of 21-49 min for breastmilk measured in infants using the ultrasound gastric antral cross sectional area measurement technique (Ewer and Yu, 1996; Yigit et al., 2008). This demonstrates that gastric emptying studies can be performed with relatively few serial measurements (immediately, 15, 30 and 60 minutes after the feed) to provide sufficient data for the analysis. This shortened duration of examination may reduce the resultant stress on the baby and make the examination more acceptable in both research and clinical settings.

Ultrasound imaging during this study provided the unique opportunity to assess gastric contents and determine the existence of any relationship between milk composition
and gastric emptying in infants for the first time. The post-feed gastric contents were visualized by ultrasound as an echogenic (white) image with a clear outline and an anechoic (black) image (Figure 5.1). Since milk curdles in the stomach due to aggregation of casein micelles at the lower gastric pH and also by the hydrolytic action of stomach proteases and this results in the separation of milk components into curd (semi solid phase that trapped casein and lipids) and aqueous portion (Mahe et al., 1996; Chatterton et al., 2004). Therefore, the echogenic (white) and anechoic (black) image are considered to be the curd and the fluid portion of the feed respectively. The ultrasonic assessment of curd formation has been previously visualized in preruminant calves where, after feeding a calf clotting milk replacer, the curd was visualized as an echogenic image and whey (or fluid portion of the feed) as an anechoic image in the abomasum (Miyazaki et al., 2009), which is similar to our results. In this study, the concentration of casein and ratio of whey to casein was found to be different in the four groups of observed post-feed stomach echogenicity, which could be due to the formation of curd that contains casein and trapped lipids (Karkiner et al., 2003). The mixed echogenicity pattern, which shows both the fluid and the curd, has the highest casein concentration and was the most frequently observed pattern compared to others. However, we didn’t find any relationship between the differences in the curd density or volume and the casein concentration, which may suggest that the intra-gastric curding is also dependent on intra-gastric conditions such as gastric pH (Chatterton et al., 2004) and requires further investigation.

In our study, no association was found between the macronutrient (fat, lactose, protein) and energy content of breastmilk and the gastric emptying, which is in contrast to reports showing that the nutrient content of formula influences the GER
such that high fat, carbohydrate, protein content and high caloric density of formula delay gastric emptying (Siegel et al., 1984; Siegel et al., 1985). Nutrient supplemented (fortified) breastmilk, which is widely recommended for pre-term infants to improve their growth and development, increases the caloric density of EBM significantly and also changes its composition (Kuschel and Harding, 2004; McClure and Newell, 1996). Both of these factors are known to delay gastric emptying in infants possibly as a result of activation of small bowel receptors (Siegel et al., 1984). Despite these reported effects most of the previous studies (McClure and Newell, 1996; Gathwala et al., 2008; Yigit et al., 2008) have not shown a difference in gastric emptying between fortified and unfortified breastmilk therefore concluding that fortifier is unlikely to affect the feed tolerance in low birth weight infants. This is consistent with our results where we have not demonstrated an effect of milk composition and energy content on gastric emptying. There was however, one study (Ewer and Yu, 1996) that found the addition of fortifier significantly slowed gastric emptying to almost twice that with EBM alone in 11 preterm infants. The fortifier used in that study (Ewer and Yu, 1996), increased the nutritional content of the milk by about 25%, which was almost twice that of other fortifiers, and is probably the reason for the slowed gastric emptying observed. Our results corroborate with these fortifier studies and suggest that breastmilk has a prokinetic property, which accelerates the gut motility, and the inhibition of gastric emptying in the presence of such activity may be possible only when the caloric value of the feed is sufficient to activate the small bowel receptors (McClure and Newell, 1996).

The limitations of this study include the small number of participants and the gastric emptying of only one feed. Although we didn’t see any effect of energy content of milk
or, an effect of individual milk proteins (whey and casein), there was higher curd volume and density exhibited later in the emptying compared to either low or no curd volume and density at the beginning of emptying, showing the slower emptying of gastric casein coagulum from the stomach compared to the liquid fraction. This suggests that the process of curd formation in the stomach provides some resistance to gastric emptying (Lorenz, 1985), which is also reflected by the curvilinear patterns of gastric emptying (Figure 5.8).

Previous studies investigating the control of food intake in breastfed infants reported a positive association between the volume of milk intake and the pre-prandial interval, which indicates that infants determine their feed volume (Matheny et al., 1990). Furthermore, an inverse relationship has been shown between the feeding frequency (feeds per day) and the average breastfeed volume indicating that the infant who feeds less often during the day consumes larger-than-average volumes and vice versa (Kent et al., 2006; Fox et al., 2006). Although we did not measure the 24h milk intake pattern of the infants that participated in this study, our results of a positive relationship between the volume of milk taken during the feed and gastric emptying are in accordance with these studies indicating that when an infant took a large feed, which should give the sensation of fullness for longer, it is likely that the stomach distension either delays the initiation of the subsequent feed or reduces the volume of intake at the subsequent feed in breastfed infants. This suggests that the gastric emptying (via stomach distension) may regulate the appetite of breastfed infants as volume of milk taken by the breastfed infant varies throughout the day (Harris, 1997). Moreover, it has also been shown previously in adults and small animals that distension of the stomach is important in promoting the feeling of satiety and post-
meal satiety is usually associated with slower gastric emptying rates (Jackson et al., 2007; Lorenz, 1985).

In conclusion, ultrasound is a reliable non-invasive method that can accurately monitor the gastric emptying in term infants and also visualize the intra-gastric curding. The colligative property of breastmilk and volume of the feed appears to influence the gastric emptying rate and is likely to affect the satiety and consequently the regulation of food intake in infants.
CHAPTER 6 General Discussion

Stomach emptying is a rate-limiting step in the digestion and absorption of nutrients; and therefore, can be involved in the short-term control of food intake and metabolism. The composition, volume and energy density of the meal has been shown to be the major determinants in the regulation of gastric emptying in adults and preterm infants (Chapter 1, Section 1.9). In healthy humans, Calbet and MacLean (Calbet, 1997) studied the role of caloric content on gastric emptying and demonstrated a linear relationship between caloric density of the ingested meal and the rate of gastric emptying. Similarly Siegel et al. (Siegel et al., 1984) showed that progressive increments of caloric density of an infant formula from 6.5 to 24 Cal/oz (0.96 to 3.5 kJ/kg) delayed the gastric emptying in the preterm infants. Further more, studies have shown that higher quantity of protein, fat, and carbohydrate in the formula meal delayed gastric emptying (Hunt and Stubbs, 1975; Burn-Murdoch et al., 1978; Siegel et al., 1985). Type of protein has also been an important factor that affects gastric emptying of liquid formulas. Different types of whey-based formulas empty faster than casein-based formulas, even with similar osmolality, caloric or fat content (Fried et al., 1992; Billeaud et al., 1990). The presence of any relationship between breastmilk composition (including the individual milk proteins; whey and casein) and gastric emptying in the healthy term infants was assessed for the first time. In our study, no association was found either between the macronutrient (fat, total protein and lactose) and the energy content of breastmilk and the gastric emptying rate (Chapter 5, Section 5.4). The curvilinear patterns of gastric emptying in infants
(Figure 5.8) and significantly higher curd volume and density exhibited later in the emptying indicate that curd formation provides some resistance to emptying and could have a role in controlling the rate of emptying. Further more, emptying rate was found to be affected by the size of the feed, with larger feeds prolonged the emptying process (Chapter 5, Section 5.4.6).

Depending on the volume of milk intake, wide variation was observed between infants in their cue for next feed. For example, Infant 9 exhibited feeding cue within 30min after an intake of 56ml, whereas Infant 2 who took a large feed of 164ml cued for next feed well after an hour. However, their stomach was not completely emptied when they cued for next feed and 25% of the feed was still remaining in the stomach (Figure 5.8). Similarly, Bateman (Bateman, 1982a) have studied the effect of meal volume on stomach emptying in human adults, and it was found that emptying was more rapid for smaller meal (200ml, t\textsubscript{1/2} 8±2min) as compare to large volume meal (500ml, t\textsubscript{1/2} 15±3min) of orange cordial. Lorenz et al. (Lorenz, 1985) have shown a significant relationship between the volume dependent gastric emptying and the inhibition of ingestion in suckling rats. Similarly, the positive linear relationship between the volume of milk intake and gastric emptying (Chapter 5, Section 5.4.3) in combination with previous reports of negative association between frequency of feeds and average feed volume indicate that infants also determine their intake in response to the rate of the GE and slow emptying may inhibit feeding. This also implies that the regulatory system based on internal cues, which are provided by peripheral sensors, such as stomach distension and gastric emptying rate (Harris, 1997) regulate the appetite (feeding patterns) of breastfed infants.
The absence of a suitable validated technique has restricted the evaluation of the gastric emptying function in healthy term breastfed infants. This study has shown that ultrasound is a reliable and repeatable method for the measurement of infant gastric volume, and for assessing the gastric emptying in healthy term infants in relation to breastmilk composition without requiring hospitalization (Chapter 5). The ultrasound method was validated using test-weighing technique, which demonstrated a significant linear relationship between intra-gastric volume (83±49ml) and the ingested milk (71±38ml) in 71% of the cases. Indeed the relationship became stronger after correcting for stomach residuals (Figure 5.4), which also proved the reliability of the ultrasound in establishing both the presence and absence of residuals. In addition, the duplicate measurements (longitudinal, anterioposterior and transverse diameter) were found to be highly consistent and unbiased, as reflected by a substantially higher ICC (>0.95), which demonstrates that the differences in gastric emptying between individuals was not because of the measurement technique (Table 5.1). Our results are consistent with the findings of McClellan et al. (McClellan et al., 2010), that assessed the reliability of ultrasound measurement protocol for infant sucking and have shown that the measurements produced by both novice and experienced raters were highly consistent and agreeable. These features strengthen the potential applicability of ultrasound imaging and measurements in clinical and research settings, for non-invasive and physiological assessment of gastric emptying in infants. Further investigation is required to determine the usefulness of this technique when investigating infants with food intolerance and emptying difficulties. Ultrasound imaging was also used to assess and grade the gastric contents after a breastfeed. The echogenicity of the gastric contents were graded in comparison to the
echogenicity of the spleen, and was also used for quantitative analysis of intra-gastric curd formation (Chapter 5). This echogenicity comparison technique is normally used for ultrasound to minimize the operator’s subjective impressions in describing the body tissues in normal or diseased state. The appearance (echogenicity) of the gastric contents was often found to be different between infants and was described as hyper-echoic, hypo-echoic, mixed and snowstorm (Figure 5.1). The different echogenicity patterns were not found to be associated with fat, total protein and lactose content of the feed, however the ratio of whey to casein was different in the identified groups. The significantly different ratio of whey to casein in the identified groups further confirms the role of these proteins in intra-gastric curd formation. However, curd formation is not only dependent on the casein content. Literature from animal studies has shown that in pre-ruminant calves curd formation of milk in the abomasum was dependent on both the quality of milk and the physical (gastric) condition of the calf. The factors associated with the calf gastric condition that results in mal formation of curd include the abomasal luminal pH and the amount of chymosin (gastric proteolytic enzyme) secretion (Miyazaki et al., 2009; Okada et al., 2009). This could explain in part the reason for no relationship between the differences in the curd density and the casein concentration, and also suggests that in infants intra-gastric curding apart from casein concentration depends on other factors such as the amount of gastric secretion and gastric pH, which requires further investigation.

The overall whey protein:casein ratio of 69:31 was found in our study (Chapter 2), which is within the range of 60:40 to 70:30 reported in literature (Kunz and Lonnerdal, 1992; Lonnerdal and Forsum, 1985). However, this ratio found in mature human milk is very different from cow’s milk, which is the base of most formula milk. Several protein
compositional differences are present between human and formula milk. The bovine milk contain a very high amount of protein (approximately 4-fold) than human milk, and 80% of this protein is present as casein and 20% as whey (Chapter 1, section 1.9.2). This is in contrast to the findings of our study and previous literature, where casein represents approximately 30-40% of the total human milk protein. To our knowledge, no previous study has investigated the ultrasonic appearance of formula milk curding in infants. The difference found in the protein ratios of these two predominant sources of infant nutrition, could result in different curd density and gastric echogenicity patterns in infants. Our classification system of visualization of gastric contents (Figure 5.1) can also be used to compare the ultrasonic imaging of gastric contents of breastmilk and formula milk. In addition, further studies investigating the ultrasonic appearance of gastric curding in breastmilk and formula fed infants; in relation to the monitoring of growth patterns in these infants are recommended.

When infants digest breastmilk, they convert it into soft flocculent curd in their stomach, which leaves the stomach slowly compared to other milk components. This mechanism maintains stable digestion and absorption in infants. Without this process, digestion is inadequate and can affect the health and growth rate of the infant (Berger et al., 1979; Mahe et al., 1996). The visualization of the gastric contents (curd) and our classification system of relative post-feed evaluation of gastric echogenicity have potential clinical implication and could be used in the assessment of slow weight gain infants.

Breastmilk composition is not uniform, and varies during a feed, with the time of the day and stage of lactation. This variability in breast milk composition could potentially
influence the “self regulated” milk consumption in infants (Neville and Robert, 1995b; Dewey and Lonnerdal, 1986). However, data concerning the regulation of milk intake in infants are limited. The wide variations observe in the feeding patterns of the infants (Figure 4.1) was not associated with the significant changes in fat content during the feed and day (Chapter 4, Section 4.4.2). This together with the same patterns of variation in energy and total solids as in fat (Figure 3.3) further confirm that breastfed infants consumed about the same amount of fat, total solids and energy throughout the day regardless of their feeding behaviour (Kent, 2007). The significant inverse relationship between the total protein intake, as well as whey and casein intake with the breastfeeding frequency (Chapter 4, Section 4.4.4) together with the observed curvilinear patterns of emptying (Figure 5.8) suggest that whey and curd proteins may have a role in the regulation of milk intake and thus body weight. These results (Chapter 4, Section 4.4.4) also highlight the importance of monitoring the milk protein composition together with milk intake in infants during the clinical treatment of low birth weight infants.

Characterization of short-term variations in breastmilk composition has sampling implications. The short-term changes which includes the changes during a feed or expression, circadian changes, between breast differences and the changes within three consecutive weeks in milk composition has been characterized (Chapter 2 and 3). No short-term changes were observed in the concentration of lactose, total protein, whey and casein (Figure 2.3 and Figure 3.1), suggest that it is not necessary to account for short-term changes in these constituents in nutritional and physiological studies. Therefore, a single breastmilk sample taken at any time during the feed can be used to estimate the concentration of these components for a mother. The consistent
between breast differences in protein concentration observed both during the 24h breastfeeding period and the expression sessions within some individuals (Figure 2.4 and 3.2) indicate that samples should be taken from both breasts of an individual mother. Short-term changes observed in fat content were consistent with the previous findings. However, we found that the mean 24h fat content was lower than the mean expression fat content (Figure 3.4), which reinforces that fat content determined from the samples collected over the 24h period should be used to obtain the representative measures of infant energy intake.

Wide variability was observed in breastmilk composition particularly the whey and casein between mothers (Chapter 3, section 3.4.2 and Chapter 2, section 2.4.4). The differences in milk individual proteins and composition between mothers supports the idea that each mother is an individual and has a characteristic breastmilk protein profile. The question remains as to whether these differences are of particular advantage to the needs of her own infant. Therefore, it is possible that the recommendations of early supplementary feeding for infants based on their growth velocity and on the assumed average concentration of mature breastmilk could be improved by measurement of the actual composition of the mother’s milk.

Nutritional status of the lactating mothers doesn’t affect the milk production and gross composition (lactose, fat and protein); however, specific factors such as vegan diet have been shown to affect nutrient adequacy of breast milk (Butte et al., 1984c; Mangels and Messina, 2001). Whitehead (Whitehead, 1979) demonstrated seasonal variation in food energy intake, body weight and milk output in rural Gambian women. Thus, under certain conditions, breastmilk production and potentially milk composition is influenced by maternal factors. The lactating mothers participated in
our studies were well nourished, however, the dietary intake of the participating mothers were not recorded. Therefore, in addition to short-term variations in milk composition (Figure 3.1) and 24h infant feeding patterns (Figure 4.1) studied in this thesis, further studies are recommended to investigate the maternal intake (type of food, amount of food, interval between meals) and sleep patterns over a 24hr cycle to provide information regarding maternal variability in breastmilk composition.

While the compositional qualities of infant formula are known to affect gastric emptying, the lack of association between gastric emptying and breastmilk energy content suggest the possible role of peptide hormones (such as leptin, ghrelin, melanocortins etc), which are associated with energy intake and expenditure. These hormones have been found in maternal milk (Palou and Pico, 2009; Fride et al., 2005), and have a role in the regulation of intake in breastfed infants. The study in rats showed that the supplementation of leptin during suckling period resulted in a decrease in food intake, and protected against overweight in adulthood (Palou and Pico, 2009). Further research investigating the role of these interacting factors in the regulation of appetite and energy intake is required, in order to understand the early metabolic programming in infants. Metabolic programming is defined as the stimulus or insult that occurred during critical period of development and has long-term effects (Lucas, 1991). Therefore, either the nutritional imbalance or the different gastric response to different feeding modes could have the potential to affect metabolism later in life leading to childhood obesity and metabolic syndrome. Further more, the differences observed in casein and whey intake (Chapter 4, Section 4.4.4), may contribute to the differences in growth patterns of infants. Since there is strong evidence that breastfed infants regulate their intake of breastmilk (Dewey and
Lonnerdal, 1986), future longitudinal studies are required to investigate the influence of the intake whey and casein on linear growth in breastfed infants.
REFERENCES


141


Yu Y, South T and Huang XF. (2009). Inter-meal interval is increased in mice fed a high whey, as opposed to soy and gluten, protein diets. Appetite 52: 372-379.