HAROKOPIO UNIVERSITY

SCHOOL OF HEALTH SCIENCE AND EDUCATION

DEPARTMENT OF NUTRITION AND DIETETICS

ASSESSMENT OF NUTRITION, GROWTH AND DEVELOPMENT OF INFANTS AND TODDLERS: THE EFFECT OF DIETARY COMPOSITION IN PROTEIN AND LIPIDS

Doctoral Thesis

Evangelia Karaglani, MSc

Athens, 2023

ΧΑΡΟΚΟΠΕΙΟ ΠΑΝΕΠΙΣΤΗΜΙΟ

ΣΧΟΛΗ ΕΠΙΣΤΗΜΩΝ ΥΓΕΙΑΣ ΚΑΙ ΑΓΩΓΗΣ

ΤΜΗΜΑ ΕΠΙΣΤΗΜΗΣ ΔΙΑΙΤΟΛΟΓΙΑΣ – ΔΙΑΤΡΟΦΗΣ

ΑΞΙΟΛΟΓΗΣΗ ΑΝΑΠΤΥΞΗΣ ΚΑΙ ΘΡΕΨΗΣ ΒΡΕΦΩΝ ΚΑΙ ΝΗΠΙΩΝ: Η ΕΠΙΔΡΑΣΗ ΤΗΣ ΣΥΣΤΑΣΗΣ ΤΗΣ ΔΙΑΤΡΟΦΗΣ ΣΕ ΠΡΩΤΕΪΝΗ ΚΑΙ ΛΙΠΑΡΑ

Διδακτορική Διατριβή

Ευαγγελία Καραγλάνη, MSc

Αθήνα, 2023

HAROKOPIO UNIVERSITY

SCHOOL OF HEALTH SCIENCE AND EDUCATION
DEPARTMENT OF NUTRITION AND DIETETICS

Declaration of Committee

Ioannis Manios (Supervisor)
Professor, Department of Nutrition and Dietetics, School of Health Science and Education, Harokopio University, Greece

George Chrousos (Examiner)
Professor Emeritus, First Department of Pediatrics, School of Medicine, University of Athens, Greece

Evangelia Charmandari (Examiner)
Professor, First Department of Pediatrics, School of Medicine, University of Athens, Greece

Maria Skouroliakou (Examiner)
Associate Professor, Department of Nutrition and Dietetics, School of Health Science and Education, Harokopio University, Greece

Paraskevi Xepapadaki (Examiner)
Associate Professor, Allergy Department, 2nd Pediatric Clinic, University of Athens, Greece

Andriana Kaliora (Examiner)
Associate Professor, Department of Nutrition and Dietetics, School of Health Science and Education, Harokopio University, Greece

Constantine Tsigos (Examiner)
Professor, Department of Nutrition and Dietetics, School of Health Science and Education, Harokopio University, Greece

The acceptance of the PhD Thesis from the Department of Nutrition and Dietetics of Harokopio University does not imply the acceptance of the author's point of view.

Ethics and Copyright Statement

I, Evangelia Karaglani, hereby declare that:

1) I am the owner of the intellectual rights of this original work and to the best of my knowledge, my work does not insult persons, nor does it offend the intellectual rights of third parties.

2) I accept that Library and Information Centre of Harokopio University may, without changing the content of my work, make it available in electronic form through its Digital Library, copy it in any medium and / or any format and hold more than one copy for maintenance and safety purposes.

3) I have obtained, where necessary, permission from the copyright owners to use any third-party copyright material reproduced in the master thesis while the corresponding material is visible in the submitted work.

To my beloved first-born son, Efthimis

“Motivation is like toothpaste. At the end of the tube, you have to squeeze harder.”*

***Source: inktales**

Acknowledgements

Words cannot express my gratitude to my supervisor and mentor, Prof. Yannis Manios, for his invaluable patience, guidance and support during this journey but also throughout my years as a researcher. I also could not have undertaken this journey without the members of my committee, Prof. George Chrousos and Prof. Evangelia Charmandari, who generously provided knowledge and expertise.

I am also grateful to my colleagues and officemates (past and present) for sharing their experience and providing their valuable guidance to me all these years, but also for their moral support. Among them, special thanks are owed to: Prof. Odysseas Androutsos and Prof. George Moschonis, Dr. Christina Mavrogianni and Dr. Christina-Paulina Lambrinou. Thanks should also go to the research assistants and study participants of the current work, who impacted and inspired me.

Lastly, I would be remiss in not mentioning my family: my parents, Stavros and Mina, and my sister, Makrina, who has always been inspirational to me; and especially my husband, Giannis, who is always by my side, pushing me and motivating me to go one step further each time. Their belief in me has kept my spirits and motivation high during this process.

TABLE OF CONTENTS

Abstract in Greek

Οι σύγχρονες μέθοδοι σύνθεσης και παρασκευής υποκατάστατων γάλακτος βρεφικής ηλικίας (ΥΓΒΗ) προσπαθούν να μιμηθούν τα συστατικά του μητρικού γάλακτος (ΜΓ) ώστε να επιτύχουν όχι μόνο τις θρεπτικές του ιδιότητες, αλλά και τις άλλες φυσιολογικές λειτουργίες που παρέχονται από το ΜΓ σε ένα παιδί που θηλάζει. Η παρούσα Διδακτορική Διατριβή έχει σκοπό να διερευνήσει τις επιδράσεις της χρήσης λίπους αγελαδινού γάλακτος (ΛΑΓ) στο μίγμα λιπαρών ΥΓΒΗ στους σάπωνες λιπαρών οξέων των κοπράνων, στην απέκκριση ασβεστίου και στα χαρακτηριστικά των κοπράνων υγιών βρεφών, καθώς επίσης να διερευνήσει τις επιδράσεις ενός ΥΓΒΗ με μερικώς υδρολυμένη πρωτεΐνη στην αύξηση και σε παραμέτρους άνεσης του πεπτικού συστήματος υγιών βρεφών σε σχέση με ένα κοινό ΥΓΒΗ με άθικτη πρωτεΐνη. Δύο κλινικές μελέτες διεξήχθησαν:

(α) Η μελέτη Little Panda ήταν μια διπλά-τυφλή τυχαιοποιημένη διασταυρούμενη κλινική μελέτη που διεξήχθη με υγιή βρέφη αποκλειστικώς σιτιζόμενα με ΥΓΒΗ, τα οποία τυχαία έλαβαν είτε ένα ΥΓΒΗ με βάση τα ΛΑΓ (50% ή 20% ΛΑΓ) είτε ένα ΥΓΒΗ με 100% φυτικά λιπαρά (ΦΛ) σε διασταυρούμενο σχεδιασμό 2x2-εβδομάδων. Στο τέλος κάθε μίας περιόδου παρέμβασης δύο εβδομάδων, συλλέχθηκαν δείγματα κοπράνων για ανάλυση λιπαρών οξέων, σαπώνων λιπαρών οξέων και απέκκρισης ασβεστίου, καθώς και αξιολόγηση της συνεκτικότητας τους. Οι ομάδες ΛΑΓ δεν έδειξαν καμία διαφορά στο παλμιτικό οξύ που απεκκρίθηκε στα κόπρανα συγκριτικά με την ομάδα ΦΛ, ενώ παρατηρήθηκαν μειωμένοι σάπωνες παλμιτικού οξέος, συνολικών λιπαρών οξέων και μειωμένη απέκκριση ασβεστίου. Επιπλέον, η ομάδα 50% ΛΑΓ έδειξε μια ευνοϊκή χαμηλότερη μέση βαθμολογία συνοχής κοπράνων συγκριτικά με την ομάδα ΦΛ. Συμπερασματικά, η χρήση ΛΑΓ στα ΥΓΒΗ μπορεί να αποτελέσει μια ενδιαφέρουσα προσέγγιση για τη βελτίωση της άνεσης του πεπτικού συστήματος και των χαρακτηριστικών των κοπράνων υγιών βρεφών, που δικαιολογεί περαιτέρω έρευνα.

(β) Η μελέτη SHIFT ήταν μια διπλά-τυφλή τυχαιοποιημένη κλινική μελέτη μη-κατωτερότητας που διεξήχθη με υγιή βρέφη αποκλειστικώς σιτιζόμενα με ΥΓΒΗ, τα οποία τυχαία έλαβαν είτε ένα ΥΓΒΗ με μερικώς υδρολυμένη πρωτεΐνη ορού γάλακτος (ΜΥΠ) είτε ένα κοινό ΥΓΒΗ με άθικτη πρωτεΐνη (ΑΠ) για τρεις μήνες, κατά τη διάρκεια των οποίων αξιολογούνταν μηναία ως προς την αύξηση (βάρος, μήκος, περίμετρος κεφαλής και τα Z-scores αυτών) και την άνεση του πεπτικού συστήματος (συνεκτικότητα κοπράνων και μικρά πεπτικά προβλήματα). Η καθημερινή

πρόσληψη βάρους ήταν παρόμοια στις δύο ομάδες με το κατώτερο όριο του 95% διαστήματος εμπιστοσύνης πάνω από το περιθώριο μη-κατωτερότητας -3 γρ./ημέρα. Καμία διαφορά δεν παρατηρήθηκε ανάμεσα στις δύο ομάδες σε άλλες παραμέτρους αύξησης (βάρος, μήκος, περίμετρος κεφαλής, δείκτης μάζας σώματος και τα Z-scores αυτών) σε καμία χρονική στιγμή. Επιπλέον, καμία διαφορά δεν παρατηρήθηκε στο συνολικό σκορ του Ερωτηματολογίου Γαστρεντερικών Συμπτωμάτων Βρεφών (ΕΓΣΒ) ανάμεσα στις δύο ομάδες. Και οι δύο ομάδες έδειξαν καλά αποτελέσματα πεπτικής άνεσης με καμία διαφορά σε κανένα στοιχείο του ΕΓΣΒ σχετικά με παλινδρόμηση, κλάμα ή ανησυχία. Καμία διαφορά στη συχνότητα κενώσεων δεν βρέθηκε ανάμεσα στις δύο ομάδες, αλλά η κατανάλωση ΜΥΠ οδήγησε σε μεγαλύτερα και πιο χαλαρά κόπρανα. Συνολικά, παρά ορισμένες διαφορές στη συνεκτικότητα των κοπράνων, το χρώμα και τον όγκο τους, η συνολική πεπτική άνεση που αναφέρθηκε ήταν συγκρίσιμη μεταξύ των δύο ομάδων. Συνεπώς, μπορεί να συναχθεί το συμπέρασμα ότι το ΥΓΒΗ με μερικώς υδρολυμένη πρωτεΐνη υποστηρίζει την επαρκή αύξηση σε υγιή βρέφη, καθώς και τα δύο ΥΓΒΗ προωθούν καλή γαστρεντερική άνεση.

Λέξεις κλειδιά: βρεφική διατροφή, αύξηση, βρεφικά γάλατα, λίπος αγελαδινού γάλακτος, υδρολυμένη πρωτεΐνη.

Abstract in English

Modern formulating and manufacturing processes of supplemental infant formulas (IFs) try to copy and imitate the components of human milk (HM) to achieve not only its nutritional properties, but also other physiological functions that are provided by HM to a breastfed child. The present Doctoral Thesis aimed to investigate the effects of bovine milk fat (MF) used in the fat blend of IFs on stool fatty acid soaps, calcium excretion and stool characteristics of healthy infants, as well as investigate the effects of a partially hydrolysed IF on growth and digestive comfort parameters in healthy infants as compared to a standard IF with intact protein. Two clinical trials were performed:

(a) Little Panda study was a double-blind randomized cross-over trial conducted with healthy formula-fed infants who were randomly allocated to receive either a MF-based formula (50% or 20% MF) or a 100% vegetable fat (VF) formula in a 2x2-week cross-over design. At the end of each two-week intervention period, stool samples were collected for fatty acids, fatty acid soaps and calcium excretion analysis and stool consistency assessment. MF-based groups showed no significant difference in palmitic acid lost in stools compared to VF group, although reduced stool palmitate soaps, total fatty acid soaps and calcium excretion were observed. Furthermore, the 50% MF group showed a favourable lower mean stool consistency score compared to the VF group. In conclusion, the use of MF in IF could be an interesting approach to improve gut comfort and stool characteristics in healthy infants, warranting further research.

(b) SHIFT study was a double-blind randomized non-inferiority trial conducted with healthy formula-fed infants who were randomly allocated to receive either a partially hydrolysed whey IF (pHF) or a standard IF with intact protein (IPF) for three months, during which they were evaluated monthly on growth (weight, length, head circumference and their Z-scores) and gut comfort (stool consistency and minor digestive issues). Daily weight gain was similar in both groups with the lower bound of 95% confidence interval above the non-inferiority margin of -3 g/day. No differences were observed between the two groups in other growth outcomes (infants' weight, length, head circumference, body mass index, and their Z-scores) at any time point. Furthermore, no differences were observed in the overall Infant Gastrointestinal Symptoms Questionnaire (IGSQ) score between the two groups. Both groups showed good digestive comfort outcomes, with no differences in any of the IGSQ items related to reflux, crying or

fussiness. No difference in defecation frequency was seen between the two groups but pHF consumption resulted in larger and looser stools. Overall, despite some differences in stool consistency, volume and colour, the overall digestive comfort reported was comparable between the two groups. Therefore, it can be concluded that the partially hydrolysed whey IF supports adequate growth in healthy infants and both formulas promote good gastrointestinal comfort.

Keywords: infant nutrition; growth; infant formula; milk fat; protein hydrolysate

List of figures

List of tables

ABBREVIATIONS

AE	Adverse event
AISS	Amsterdam Infant Stool Scale
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
AST	All subjects treated
BF	Breast-fed
BMI	Body mass index
Ca	Calcium
CMPA	Cow's milk protein allergy
CS1	Cross-over study 1
CS2	Cross-over study 2
EFSA	European Food Safety Authority
eHF	Extensively hydrolysed formula
FA	Fatty acid
FDA	Food and Drug Administration
FF	Formula-fed
GCP	Good Clinical Practice
GI	Gastrointestinal
GOS	Galacto-oligosaccharides
HM	Human milk
HMO	Human milk oligosaccharides
ICH	International Conference on Harmonization
IF	Infant formula
IGSQ	Infant's Gastrointestinal Symptoms Questionnaire
IPF	Intact protein formula
ITT	Intention to treat
MEC	Medical Ethics Committee
MF	Milk fat
MMRM	Mixed models repeated measures

PA	Palmitic acid
pHF	Partially hydrolysed formula
PI	Principal investigator
PP	Per protocol
QPGS-RIII	Questionnaire on Paediatric Gastrointestinal Symptoms-ROME III (infant version)
SAE	Serious adverse event
SAP	Statistical analysis plan
SD	Standard deviation
TAG	Triacylglycerol
VF	Vegetable fat
WHO	World Health Organization

1. INTRODUCTION

1.1 INFANT NUTRITION, GROWTH AND DEVELOPMENT

Human milk (HM) represents the optimal nutrition for infants after birth and during whole infancy. Progressive discoveries over the last decades of its particular components and their physiological properties have allowed better understanding of the nutritional functions, as well as a whole other spectrum of non-nutritional functions that can be distinguished in HM, including immunomodulatory and other physiological activities (Andreas et al., 2015). In terms of protein content, for example, HM contains numerous bioactive proteins and peptides including antimicrobial and immune-modulating factors, enzymes, hormones and growth factors. Also, regarding lipid content, HM is rich in some fatty acids (FAs) essential for brain development, while it contains several non-lactose carbohydrates that play an important role in resistance to infection (Dewey, 2001). Notably, the composition of HM is dynamic and changes across the period of lactation in response to many factors, matching the infant's nutritional needs according to its age and other characteristics and ensuring healthy normal growth and development (Lonnerdal, 1986; Lonnerdal et al., 1976; Michaelsen et al., 1990).

Revealing the function and importance of the particular components of HM has allowed for improvement of modern supplemental milk formulas for infants who, for various reasons, cannot be breastfed. Modern formulating and manufacturing processes of infant formulas (IFs) try to copy and imitate the components of HM to achieve not only its nutritional properties, but also the other physiological functions that are provided by HM to a breastfed child, as described above (Koletzko et al., 2011).

1.1.1 Human milk and infant formula composition

1.1.1.1 Lipids

Lipids are the largest source of energy in HM, contributing 40–55 % of its total energy. Triacylglycerols (TAGs) in HM provide approximately 50 % of the energy as well as essential FAs important for the overall development of the infant (Delplanque et al., 2015; Koletzko et al., 2001; Miles and Calder, 2017). The remainder predominantly consists of diacylglycerols, monoacylglycerols, free FAs, phospholipids and cholesterol. HM contains over 200 FAs; however, many of these are present in very low concentrations, with others dominating (Koletzko et al.,

1988). Palmitic acid (C16:0; PA), one of the major saturated FAs in HM (representing approximately 20–25 % of total FAs), is predominantly esterified at the SN-2 position of TAGs (i.e. SN-2-palmitate) in HM (Andreas et al., 2015; Koletzko et al., 2001; Marie Straarup et al., 2006). Studies over the last two to three decades have provided increasing evidence that the SN-2-predominant positioning of PA in HM TAGs promotes the absorption of both PA and calcium in term and preterm infants (Bar-Yoseph et al., 2016; Miles and Calder, 2017; Petit et al., 2017).

The majority of IFs use a blend of vegetable oils as a source of fat. Compared to HM fat, in which 70–88 % of the PA is esterified at the SN-2 position, commonly used vegetable oils have lower percentage of PA in the SN-2 position of TAGs (10–20 %) (Marie Straarup et al., 2006). Therefore, vegetable fat (VF) blends consist of TAGs with PA predominantly bound to the SN-1 and SN-3 positions (Havlicekova et al., 2016; Marie Straarup et al., 2006) (Figure 1.1). During digestion, PA at the SN-1,3 positions is released as free PA. In the alkaline environment of the small intestinal lumen, free PA interacts readily with cations (e.g. calcium) to form insoluble soaps (Innis, 2011; Lindquist and Hernell, 2010) that are associated with hard stools, gut discomfort and decreased absorption of PA and minerals by the infant (Innis, 2011; Petit et al., 2017; Quinlan et al., 1995). Increasing the ratio of SN-2 to SN-1 and SN-3 palmitate in IF could ensure higher absorption of fat and minerals (calcium), as well as lead to reduced formation of insoluble soaps, thereby, minimizing gut discomfort.

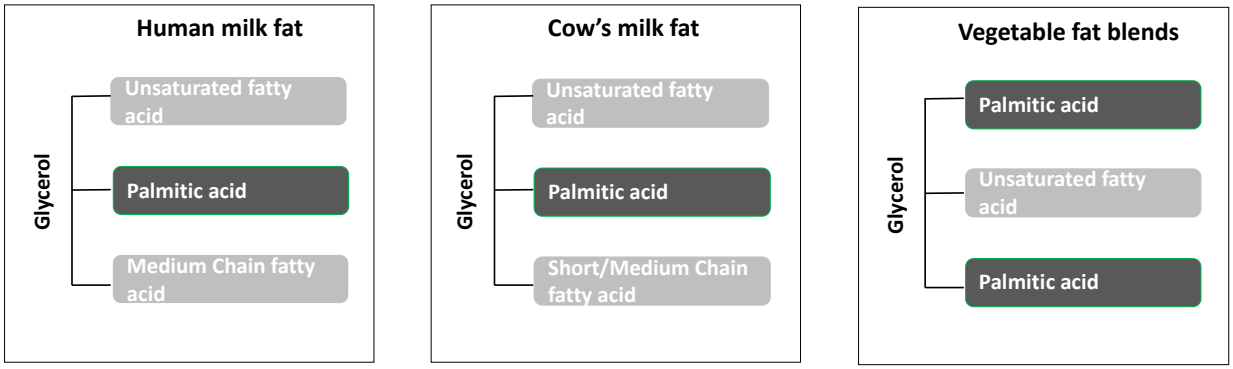

Figure 1-1. Distribution of SN-2-palmitate at the glycerol backbone of TAGs in specific types of fat.

Synthetic structured TAGs have been developed with higher proportion of PA in the SN-2 position (ranging from 35.9–74 %) and lower levels of PA at the SN-1 and SN-3 positions. Favourable effects of IF containing such synthetic TAGs on FA, calcium absorption and stool consistency have

been reported in healthy infants by several studies (Bar-Yoseph et al., 2016; Béghin et al., 2019; Carnielli et al., 1996, 1995; Kennedy et al., 1999; López-López et al., 2001; Lucas et al., 1997; Nowacki et al., 2014; Yao et al., 2014). Bovine milk fat (MF) is naturally higher in SN-2-palmitate than VFs, with a level of approximately 40 % (Havlicekova et al., 2016; Innis, 2011; Petit et al., 2017) and a higher ratio of SN-2 vs SN-1,3 palmitate. Furthermore, MF shows comparable TAG structures to those in HM fat (Petit et al., 2017) (Figure 1.1). Therefore, using MF in the development of IF may enable mimicking the composition and structure of HM fat, potentially leading to a higher absorption of PA and calcium, less soap formation and softer stools in comparison to IF containing VF only.

1.1.1.2 Protein

The infant's first year of life is a critical time characterized by rapid growth and development which are crucial for long-term well-being. Indicatively, an infant's body weight doubles by the age of six months (Nutten, 2016). Therefore, the rapid growth of the baby must be supported by a high rate of protein synthesis. In the first month of life, infants need around 3.5 times as much protein per kilogram of body weight as an adult, at the age of four to six months infants still need more than 60 %, and at six to 12 years around 40 % more protein than adults per kilogram of body weight (Nutten, 2016). Both total protein content and concentrations of individual proteins in HM change throughout the first year of lactation to match the needs of the infant.

Today, besides HM, IF is the only other milk product considered nutritionally acceptable for infants under the age of one year (as opposed to unmodified cow's or goat's milk). Protein sources and processes of IF have been modified along the years to optimize both the quality and the quantity of proteins in IFs in order to be closer to the composition but also the functional outcomes of breast milk. Besides ensuring healthy growth and development of formula-fed infants, specific IFs have also been designed for specific needs, by modifying their protein component (Nutten, 2016). As an example, three different types of IFs have been designed for the prevention and management of cow's milk protein allergy (CMPA). One way to decrease allergenicity of proteins is to disrupt the sequence or to modify the conformation of the allergenic epitopes by enzymatic hydrolysis. According to the process and degree of hydrolysis, different types of formulas can be obtained: partially (pHF) or extensively hydrolysed formula (eHF) or amino acid-based formulas (L. Barrera et al., 2021) (Figure 1.2). The differentiation between eHF

and pHF is mostly established by the molecular weight profile and clinical demonstration of reduced allergenicity.

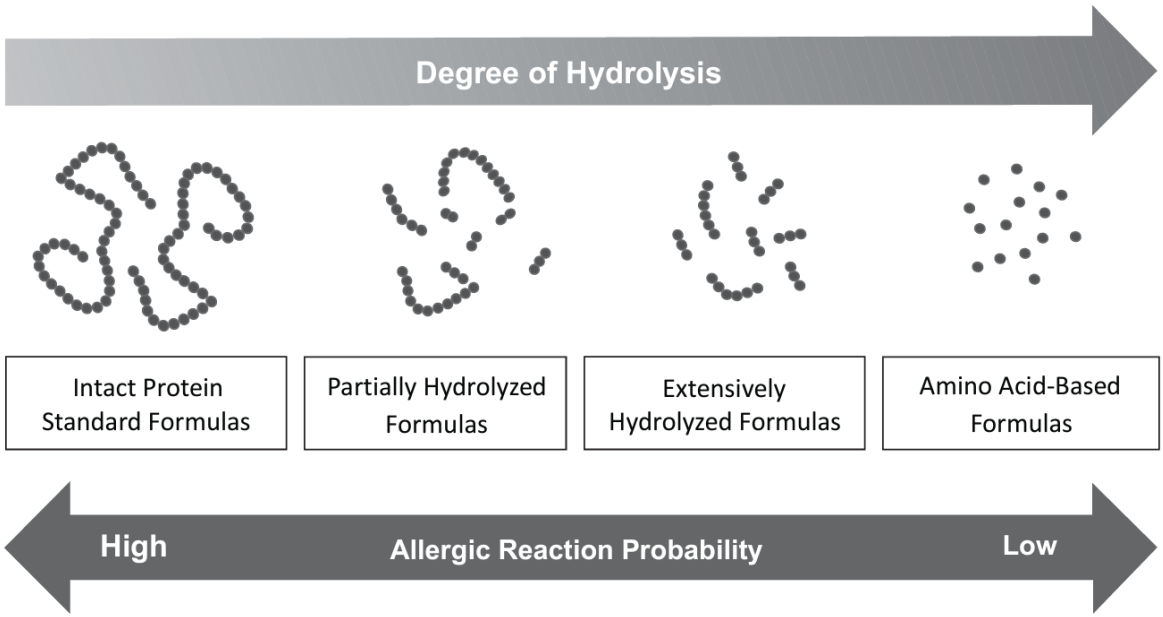

Figure 1-2. Protein structure in infant formulas and risk for allergic reaction in infants with CMPA (*adapted by Barrera et al. 2021*).

pHFs are not intended for infants with established CMPA. Instead, they have been developed for infants at high risk of allergy (based on family history) to prevent onset of the disease. This is most probably relying on the induction of oral tolerance to cow's milk protein mediated through the interaction of specific peptides with the immune system (Fritsché et al., 1997; Pecquet et al., 2000). Specific pHFs have been clinically proven to prevent atopic dermatitis when used during the first four months of life in infants with a family history of allergy (Alexander and Cabana, 2010; Szajewska and Horvath, 2010; Von Berg et al., 2003). In the absence of breastfeeding, different paediatric international organizations recommend using the clinically documented pHFs when breastfeeding is not possible.

1.1.1.3 Carbohydrate

A huge variety of different and complex carbohydrates are present in HM, contributing 7 % of its total energy. Among them, lactose, a disaccharide consisting of glucose covalently bound to galactose, is dominating (Berger et al., 2020), corresponding to the high energy demands of the human brain. Lactose also supports the absorption of minerals and calcium. However, there is emerging evidence that additional carbohydrate fractions play an important role in determining

early infant health. These include human milk oligosaccharides (HMO), a family of structurally diverse unconjugated glycans with one or more residues that dictate their distinct function, as well as fructose (Andreas et al., 2015; Coppa et al., 1993). HMO are indigestible by the infant, but their function instead is to nourish the gastrointestinal (GI) microbiota and they possess anti-infective properties against pathogens in the infant GI tract. Furthermore, HMO have been found to play a role in infant growth and body composition (Berger et al., 2020). HM contains a high concentration and unique structural diversity of HMO. Studies have shown that breastfed infants have a more stable and uniform population of oligosaccharides compared with formula-fed ones (Bezirtzoglou et al., 2011; Musilova et al., 2014). Therefore, it is important that IFs are supplemented with probiotics and prebiotics. Indicatively, supplementation of formula with probiotics represents a key strategy to reduce the incidence and severity of diarrhoea in infants (Chassard et al., 2014; Martin et al., 2016).

1.1.1.4 Vitamins, minerals and other nutrients

HM contains adequate amounts of most vitamins to support normal infant growth, except for vitamins D and K (Martin et al., 2016). Infants who are exclusively breastfed are at risk for vitamin D deficiency, inadequate bone mineralization and conditions such as rickets. However, overall sun exposure also plays a role in the overall risk of vitamin D deficiency in breastfed infants. Formula-fed infants often have higher serum concentration of vitamin D metabolites than breastfed infants (Martin et al., 2016).

Minerals in HM contribute to a variety of physiological functions, as they form essential parts of many enzymes and are of biological importance to molecules and structures. HM and bovine milk have comparable content of minerals. Over the decades, many other bioactive components have been identified in HM, including hormones, growth factors and immunological factors (Jiang, 2014; Martin et al., 2016).

1.1.2 Effects of specific types of infant formulas on health outcomes

1.1.2.1 SN-2-palmitate content

According to the available evidence from clinical trials summarized below and as also illustrated in Figure 1.3, several favourable biological effects of adding SN-2-palmitate to IF have been reported.

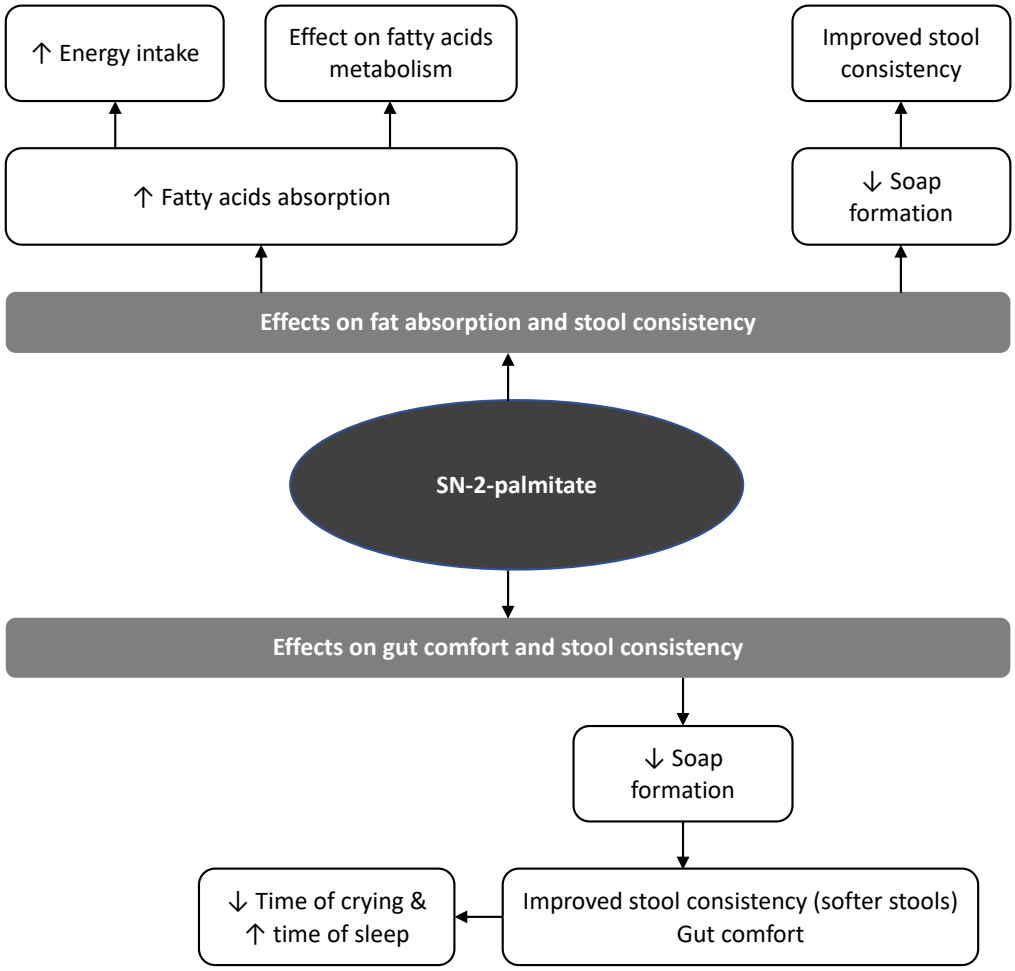

Figure 1-3. Potential health effects of SN-2-palmitate (*adjusted from Havlicekova, Z. et al. 2016*).

Influence of SN-2-palmitate on the absorption of fat and calcium. Two older "balance studies" (Carnielli et al., 1995; Lucas et al., 1997) reported higher absorption of PA and saturated FAs, as well as lower formation of FA-calcium soaps in the intestine of infants fed with formula mimicking HM in terms of SN-2-palmitate content. Additionally, high SN-2-palmitate content in the tested formulas improved calcium absorption, thus leading to lower calcium excretion at stools and urine, as compared to the standard formulas. Recent studies, albeit with different study designs, have also reported lower excretions of PA, total FAs and insoluble calcium soaps in response to 4-12 weeks of formulas with higher (35.9 % - 50 %) SN-2-palmitate levels (Bar-Yoseph et al., 2016; Kennedy et al., 1999; López-López et al., 2001; Nowacki et al., 2014) compared to standard formulas (11.7 % - 19 % SN-2-palmitate). (Nowacki et al., 2014) observed significantly lower concentrations of PA and palmitate soaps in faeces in the high SN-2-palmitate group (PA in sn-2

position: 38.9 %) as compared to the standard formula group (PA in sn-2 position: 12.6 %), although no significant differences were observed between groups with regards to the concentrations of total FA soaps. In contrast, (Bar-Yoseph et al., 2016) reported significantly lower concentrations of faecal total FAs and FA-calcium soaps in Chinese infants that received a formula with a high content in SN-2-palmitate (PA in sn-2 position: 43 %) for six weeks as compared to the standard formula (PA in sn-2 position: 13 %). Furthermore, (López-López et al., 2001) and (Kennedy et al., 1999) reported significantly lower concentrations of PA, total FAs and FA soaps in faeces of infants that were fed for eight and 12 weeks, respectively, with formulas having a high SN-2-palmitate content (PA in sn-2 position: 44.5 % and 50 %, respectively), compared to infants that were given standard formula (PA in sn-2 position: 19 % and 12 %, respectively). Similar findings were also reported by (Yao et al., 2014) who confirmed the lower faecal concentrations of total FA soaps and palmitate soaps in infants given the high SN-2-palmitate formula (PA in sn-2 position: 35.9 %) as compared to infants fed with the standard formula (PA in sn-2 position: 11.7 %) for an intervention period of eight weeks. Interestingly, (Yao et al., 2014) also reported significantly lower concentration of total FA soaps for the high SN-2-palmitate formula group as compared to the breastfed group.

Influence of SN-2-palmitate on overall gut comfort. This has been previously assessed mainly using self-reported tools completed by parents/caregivers; either tools assessing stool consistency, and/or tools assessing incidence of minor GI issues (e.g. hard stool, constipation, diarrhoea, regurgitation, colic and crying episodes).

Several clinical trials assessing stool consistency have reported softer stools in infants provided with high SN-2-palmitate formula (i.e. PA in sn-2 position: 35.9-50 %), even approaching stool consistency observed for breast-fed infants (Kennedy et al., 1999; Litmanovitz et al., 2014; Nowacki et al., 2014; Yao et al., 2014). More recent studies in healthy term infants consuming high SN-2-palmitate formula, however, suggest that this effect is more visible with the addition of prebiotic oligofructose (Bongers et al., 2007; Nowacki et al., 2014; Yao et al., 2014). In all these previous clinical trials, parents have assessed infants' stool consistency by keeping stool diaries and using a limited number of standardized pictures of stools, while no clinical trial is available in the literature having used the more recent "Amsterdam Infant Stool Scale" (AISS). AISS seems to be more appropriate tool to use since it is an age specific, validated scale developed for use in

infants who are not yet toilet trained and can be used to describe the amount, consistency and colour of infants' stools (Ghanma et al., 2014).

GI tolerance issues have been previously assessed either by the Infant's Gastrointestinal Symptoms Questionnaire (IGSQ) (Nowacki et al., 2014; Yao et al., 2014), or by diaries kept by parents, recording the frequency of colic and/or crying episodes (Kennedy et al., 1999; Litmanovitz et al., 2014). IGSQ is a 13-item validated tool that is filled in by parents and provides a score, the higher values of which are indicative of higher GI burden (or lower GI/gut comfort) (Riley et al., 2015). In the studies by (Nowacki et al., 2014) and (Yao et al., 2014), no significant differences in the IGSQ index total score and individual scores at four and eight weeks of intervention were reported among the high SN-2-palmitate (PA in sn-2 position: 38.9 % and 35.9 %, respectively) and standard formula groups (PA in sn-2 position: 13 % and 11.7 %, respectively) and the breastfed group. Similarly, (Kennedy et al., 1999) reported no significant differences in the frequency of colic and the duration of crying episodes between the high SN-2-palmitate (PA in sn-2 position: 50 %) and standard formula groups (PA in sn-2 position: 12 %) and breastfed group, as quantitatively assessed via diaries. On the contrary, the only study so far reported significantly shorter mean total crying duration in the high SN-2-palmitate formula group (PA in sn-2 position: 44 %) as compared to the standard formula group (PA in sn-2 position: 14 %) after 12 weeks of intervention was that by (Litmanovitz et al., 2014). Although the IGSQ is a valid tool for assessment of infant GI-related behaviours, none of the previous clinical trials have used the validated Questionnaire on Paediatric Gastrointestinal Symptoms - Rome III infant/toddler version (QPGS-RIII infant/toddler) (Van Tilburg et al., 2016) that is based on the "Rome III diagnostic criteria for functional gastrointestinal disorders" (infant rumination, infant colic, infant regurgitation, infant dyschezia, cyclic vomiting syndrome, functional diarrhoea, and functional constipation).

Available studies suggest that increasing the content of SN-2-palmitate in IFs may have several beneficial physiological functions, such as positive influences on FA metabolism, calcium absorption and stool consistency. Addition of MF to an IF can help increase the SN-2-palmitate levels of the formula and potentially, confer similar favourable effects to the infants.

1.1.2.2 Protein hydrolysis

Protein sources and IF processing technologies have been modified over the past years to optimize both the quality and the quantity of proteins in IF to better suit the nutritional

requirements of infants and support more optimal growth. Protein hydrolysis, i.e., where proteins are digested into smaller fragments, peptides, or amino acids, is a frequent modification in IF, particularly those designed for special medical purposes (Yvan Vandenplas et al., 2014). Depending on the level of hydrolysis, hydrolysates can be classified as partially or extensively hydrolysed proteins.

Hydrolysate-based formulas have been mainly developed for CMPA management, as IF containing extensively or partially hydrolysed proteins are suggested to reduce the risk of developing allergic manifestations during the first four to six months of life (Alexander and Cabana, 2010; Von Berg et al., 2003). CMPA is caused by an abnormal immune reaction to cow's milk protein (Taylor, 1986). About 2-5 % of all new-borns suffer from CMPA within the first year of life (Katz et al., 2010) while 5-15 % of infants show symptoms suggestive of CMPA (Host and Halken, 2014). The best preventive measure against the development of CMPA is to provide breastfeeding. Should breastfeeding not be possible, feasible or desirable, then IF containing extremely or partially hydrolysed proteins could be provided.

Several studies demonstrated that the use of extremely hydrolysed protein fractions is effective in the management of CMPA (Halken et al., 2000; Høst and Halken, 2005; Von Berg et al., 2003) in formula fed infants. For less extensively hydrolysed protein fractions the risk reducing effect is not always clear and this should be established per product (brand). Although the risk reducing effects of partially hydrolysed formulas may be less than in extremely hydrolysed protein, additional benefits of partially hydrolysed protein products are suggested to be faster development of cow's milk protein tolerance, better taste, texture and overall palatability.

Gastro-oesophageal reflux, diarrhoea, constipation and colic are among the most common mild GI disorders in infancy and early childhood (Heine, 2008). These are not clinically diagnosed diseases, but occur commonly, causing discomfort to infants and distress to parents. With no medically indicated treatments, dietary alterations are the most appropriate approach to resolve these mild GI symptoms (Huang et al., 2021). Breast milk is the optimal option; still, for formula-fed infants (either exclusive or mixed feeding), standard cow's milk formula needs to be substituted by specific IFs. Most of these GI disorders are related to an underdeveloped GI system which is vulnerable to ingredients contained in IFs, such as proteins and lactose. Replacing intact protein with hydrolysed protein in IFs is one way to improve the GI comfort in healthy term infants, especially in the early postnatal period (Bhatia et al., 2016). Several studies have reported

lower incidence of regurgitation, increased stool frequency (Savino et al., 2005), a positive effect on constipation (Savino et al., 2006) and decrease in infantile colic episodes (Bongers et al., 2007). However, most clinical trials assessing the effects of pHF in infants with mild GI disorders included additional formula modifications besides the protein fraction (change from intact to partially hydrolysed protein), such as addition of prebiotics, PA, and decreased lactose content (Vandenplas et al., 2019). Indeed, since not all hydrolysed formulas are the same with regards to their nutrient composition, the allergenicity, tolerability, effectiveness, and clinical impact of each pHF differ, and each product should be clinically evaluated.

1.2 AIM AND OBJECTIVES

The present Doctoral Thesis aims to evaluate several nutritional factors affecting growth and development of healthy term infants, as following:

1. The effects of the nutritional composition of different infant formulas on infants' nutrient absorption and growth.

2. The effects of the nutritional composition of different infant formulas, especially regarding their protein content, on weight gain of healthy infants.

3. Whether growth of healthy infants consuming exclusively infant formula is in accordance with the WHO growth standards.

4. The effects of the type of formula consumed on growth and development (including weight, length and head circumference) of healthy infants, as well as on gastrointestinal tolerance.

The specific objectives of the three manuscripts that comprise the present Doctoral Thesis are the following:

Manuscript 1: To investigate the effect of bovine milk fat, a natural source of SN-2-palmitate, used in the fat blend of infant formulas, on stool fatty acid soaps, calcium excretion and stool characteristics of healthy term infants.

Manuscript 2: To investigate the effects of a partially hydrolysed whey infant formula on growth in healthy term infants as compared to a standard infant formula with intact protein.

Manuscript 3: To investigate the effects of a partially hydrolysed whey infant formula on digestive comfort parameters of healthy term infants compared to an intact protein formula, as well as to assess links of corresponding growth data with gastrointestinal comfort.

2. METHODOLOGY

2.1 LITTLE PANDA STUDY AND SHIFT STUDY

The present Doctoral Thesis includes two randomized clinical trials: the "Little Panda study" (manuscript 1) and the "SHIFT study" (manuscripts 2 and 3). Both studies are conducted with healthy term infants consuming IF and aim to examine infants' growth, as well as GI outcomes.

2.1.1 Little Panda study design

The Little Panda study comprises of two separate double-blind, cross-over, randomized clinical trials conducted in parallel with healthy, full-term, exclusively formula-fed (FF) infants, each one comparing a MF-based formula against a standard VF formula. The primary objective of these trials is to evaluate the excretion of PA and PA soaps in stools of healthy term infants. We hypothesized that infants fed MF-based IF had lower PA and PA soaps in stool when compared to infants fed VF-based formula. In addition, the secondary outcomes of both trials include calcium excretion in stools, stool consistency scores and other FA and FA soaps in stools.

The total duration of each cross-over study (CS1 and CS2, respectively) was six weeks, including a 2-week wash-out (or run-in) period before starting the trial, two weeks consuming the standard VF formula, followed by another two weeks consuming the MF-based formula, or vice-versa (Figure 2.1).

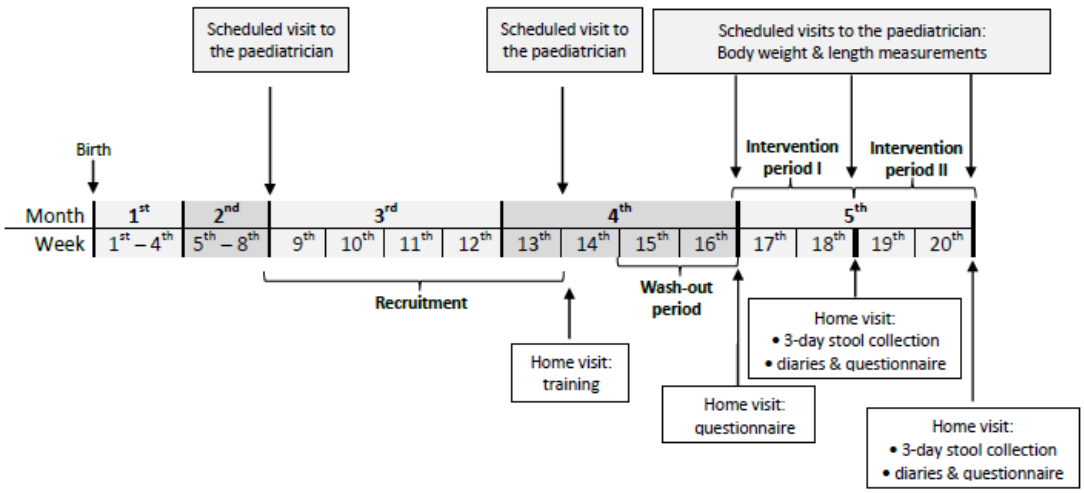

Figure 2-1. Timeline of each cross-over study in Little Panda study.

2.1.1.1 Sampling and recruitment

Sampling and recruitment were performed by paediatricians at 12 private paediatric clinics in two cities (Athens and Larissa) in Greece, during infants' routine visits to their paediatrician between 9th-14th week of age. Specifically, recruitment procedure was performed as following:

- The first contact with the parents was made at the infants' 9th week of age, during routine visit to the paediatricians, when parents were provided by the paediatrician with all information regarding the study aim and procedures and were given time to consider their child's participation in the study.
- The inclusion in the study was defined at a second contact by infants' 14th week of age, during the next scheduled visit to the paediatrician. There, any questions from the parents were clarified and the infants were screened on whether they fulfilled the inclusion criteria, in order to be included in the study.
- After providing any additional clarifications and feedback to parents, those parents still interested to participate in the study were asked to provide a signed consent form.

2.1.1.2 Inclusion and exclusion criteria

Paediatricians evaluated the appropriateness of inclusion in the study only for exclusively FF infants and not for breastfeeding ones, to avoid encouraging parents to switch to formula feeding. This evaluation was based on the following inclusion and exclusion criteria:

<u>Inclusion criteria</u>

- Full-term, healthy infants (born at gestational age ≥37 weeks).
- Appropriate for gestational age birthweight (i.e. 10th centile ≤ Birth weight ≤ 90th centile).
- Age at enrolment: between 9th-14th week.
- Exclusively formula fed infants before and during the entire intervention period.
- Parents willing and agreeing to initiate complementary feeding after the end of endpoint measurements, i.e. after the completion of the 5th month of age.

- Parents willing to collect stools and fill in all study questionnaires and diaries during the entire intervention period.
- Written informed consent.

Exclusion criteria

- Severe acquired or congenital diseases, mental or physical disorders, any symptoms of allergy (including cow's milk protein allergy – CMPA).
- No parents or siblings with documented CMPA, diagnosed by a doctor.
- Use of probiotics, antibiotics or other medication that treat or cause gastrointestinal symptoms and/or affect appetite at the time of screening or at any time throughout the study period (these infants will be considered as drop-outs).
- Use of medication(s) known or suspected to affect fat digestion, absorption and/or metabolism; nutritional supplements; suppositories; medication that may suppress or neutralize gastric acid secretion and gut mobility at the time of screening or at any time throughout the study period (these infants will be considered as drop-outs).
- Participation in another clinical trial.
- Any type of mixed feeding (i.e. combination of formula with breastfeeding in any proportion) and/or complementary feeding during the intervention.

2.1.1.3 Treatment allocation and study formulas

Upon inclusion in the study, all infants were fed the 100 % VF formula with 10.1 % SN-2-palmitate levels (total PA 24.9 %) for two weeks (wash-out period) in order to minimize the potential effects of previous feedings. Infants were then allocated to one of the cross-over studies. In each of the studies infants were randomly assigned to receive either the VF formula or a MF-based formula: i) 50 % MF + 50% VF (50MF) with 39 % SN-2-palmitate levels (total PA 18.9 %) in cross-over study 1 (CS1) and ii) 20 % MF + 80% VF (20MF) with 19.7 % SN-2-palmitate levels (total PA 26.1 %) in cross-over study 2 (CS2). Randomization into the two treatment arms per study was based on a computer-generated sequence. After 2 weeks (period I), infants were crossed over to receive the other formula for another two weeks (period II) in their respective CS1 and CS2 (Figure 2.2).

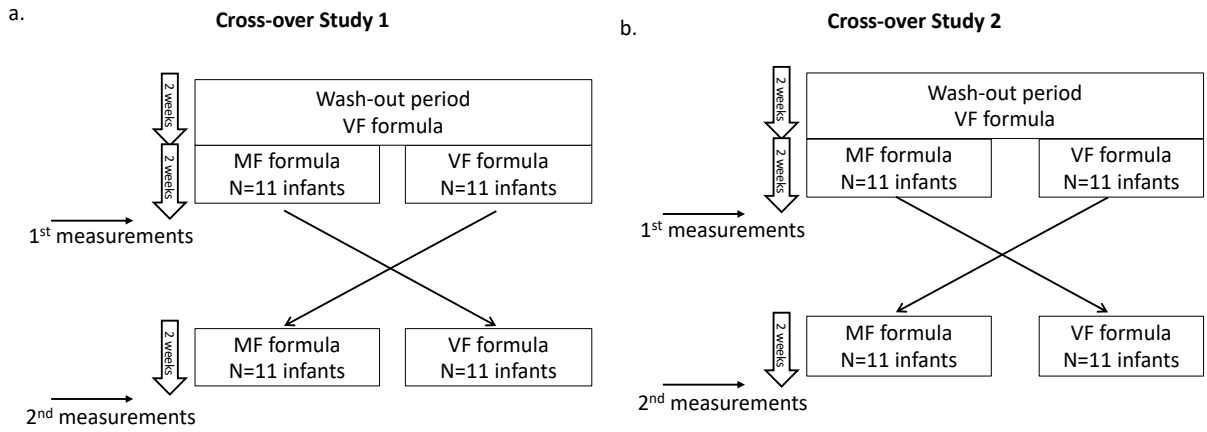

Figure 2-2. Little Panda Study flowchart and subjects' disposition.

The nutritional composition of the three study formulas was similar with the only difference being their FA profiles and percentage of SN-2-palmitate (Table 2.1). All formulas were produced in the Netherlands by FrieslandCampina and were packaged in similar blank tins of 400 g each with a specific identification code at the bottom of the tins. All study personnel, as well as parents/caregivers were blinded to the formula allocation. Sealed envelopes containing product codes were kept in the study site in the event of an emergency. The tin label included guidance for the parents on the daily volume of formula intake required by the infant, which depends upon age and weight.

Table 2-1. Composition of the Little Panda study formulas.

		Formula	
Nutrient/ingredient	**50MF**	**20MF**	**VF**
Energy (kcal/100 mL)	66	66	66
Intact protein (g/100 mL)	1.4	1.4	1.4
Carbohydrates (g/100 mL)	7.1	7.0	7.0
Galacto-oligosaccharides (g/100 mL)	0.27	0.27	0.27
Fat (g/100 mL)	3.5	3.5	3.5
Docosahexanoic acid (mg/100 mL)	6.9	6.9	6.9
Arachidonic acid (mg/100 mL)	8.3	8.3	6.9
Fatty acids; mol % of TAGs			
C12:0; Lauric acid	6.0	7.7	10.4
C14:0; Myristic acid	7.4	4.8	3.9

C16:0; Palmitic acid	18.9	26.1	24.9
C18:0; Stearic acid	5.2	4.4	3.4
C18:1; Oleic acid	36.9	42.2	39.0
C18:2; Linoleic acid	11.7	16.4	12.7
C18:3; a-Linolenic acid	1.5	1.6	1.8
C20:0; Arachidic acid	0.2	0.3	0.3
% C16:0 in sn-2 position	39	19.7	10.1
Calcium (mg/100 mL)	53	55	56

MF: milk fat; VF: vegetable fat. 50MF: 50 % MF formula; 20MF: 20 % MF formula

2.1.1.4 Stool collection and analysis

Stool samples were collected at home by parents/caregivers for three consecutive days at the end of period I and period II for analysis of their FAs, FA soaps and calcium content. Each freshly passed stool was placed in a faecal tube collector (until 30 g was collected in total), kept in a zip lock amber plastic bag and then stored in the home freezer. At the end of each intervention period, the study personnel collected the stool samples from the homes and brought them to Harokopio University. The stool samples were stored in Harokopio University in a freezer at -80°C until being transported in dry ice to Covance Laboratory, Madison, Wisconsin, USA for analysis.

2.1.1.5 Formula consumption and stool characteristics

Parents/caregivers were asked to record formula consumption using a three-day milk diary, where the timing, frequency as well as the exact amount/volume (in mL) of formula consumed was recorded during the same three days of each intervention period as stool collection. Additionally, the study personnel collected all formula tins to monitor compliance and formula consumption.

Stool characteristics assessment was performed by parents/caregivers using the validated Amsterdam Infant Stool Scale (AISS) (Bekkali et al., 2009), which assesses the consistency, amount/volume and colour of stools. For assessment of consistency, each freshly passed stool during the three-day period was evaluated and ranked accordingly on a scale of one to four (watery = 1, soft = 2, formed = 3, hard =4) and a mean score was calculated.

2.1.1.6 Safety and anthropometric assessment

Adverse events (AEs) and serious adverse events (SAEs) were recorded throughout the study and monitored by an independent paediatrician. Anthropometric indices (weight and length) were also measured following standardized procedures at screening and at the end of the run-in period, period I and period II.

2.1.1.7 Outcome Measures

The primary outcome measures to evaluate the fatty acids absorption were palmitic FAs lost in stool and palmitate soaped FAs lost in stool. The secondary outcome measures were calcium absorption and overall gut comfort, including assessment of stool consistency, volume and colour and calcium absorption.

2.1.2 SHIFT study design

The SHIFT study is a double-blind, randomized controlled trial including two study arms: a partially hydrolysed whey-based formula (pHF; test) and a standard cow's-milk formula with intact protein (IPF; control). The primary objective of this study was to evaluate the weight gain of infants consuming the whey-based pHF compared to the standard IPF over a period of three months. The secondary objective included evaluation of additional anthropometric indices at every timepoint over the period of three months. The tertiary objective was to evaluate the effect of the pHF on gastrointestinal comfort compared to the IPF.

The study was conducted with healthy, full-term, exclusively FF infants that were randomly allocated to receive one of the two formulas. The total duration of the intervention for each participant was three months where a month was defined as 30 days. Specifically, three follow-up visits were performed in total, at the following time-points: baseline +30, +60 and +90 days with an allowed deviation of +/- 2 days. The study was conducted in two cities in Greece, being Athens and Larissa, at 39 private paediatric clinics.

2.1.2.1 Sampling and recruitment

Infants were recruited from the 55th until the 80th day of age during routine visits to their private paediatricians. In more detail, recruitment was performed as following:

- The paediatrician screened for interest/ made the first contact with the parents during routine visits at any moment before the 75th day of age. Paediatricians only

approached parents of exclusively FF infants and only those that showed interest were provided with study information. Parents were then given sufficient time (± one week) to consider their child's participation in the study.

- Inclusion in the study was finalized at a second contact any time before the 80th day of age. After providing any additional clarifications and feedback to parents, those parents still interested to participate in the study were asked to provide a signed consent form.
- Upon signing the informed consent form, infants were screened on whether they fulfilled the inclusion criteria, and the baseline measurements were performed if the infant was confirmed to be eligible for participation in the study.

In all cases parents were free to withdraw their infants from the study at any time without any consequences.

2.1.2.2 Inclusion and exclusion criteria

Private paediatricians evaluated the appropriateness of inclusion of exclusively FF infants, based on the following inclusion and exclusion criteria:

Inclusion criteria

- Full-term, healthy infants (born at gestational age ≥37 weeks) in the general population
- Appropriate for gestational age birthweight (i.e. 10th centile ≤ Birth weight ≤ 90th centile)
- Boys and girls
- Age at enrolment (baseline measurement): between 55 and 80 days of age
- Exclusively formula fed two weeks before inclusion
- Exclusively formula fed during the entire intervention period
- Parents agreeing to initiate complementary feeding after finalization of the study (endpoint measurements at ~5.5 months of age)

- Being available for follow up until the age of approximately 5.5 months
- Written informed consent

Exclusion criteria

- Severe acquired or congenital diseases, mental or physical disorders including cow's milk protein allergy (CMPA), lactose intolerance and diagnosed medical conditions that are known to affect growth [i.e. GI disorders]
- Illness at screening/inclusion
- Incapability of parents to comply with the study protocol
- Participation in another clinical trial
- Unwillingness to accept the formula supplied by the study as the only formula for their child during study participation

2.1.2.3 Treatment allocation and study formulas

Upon inclusion in the study, participants were randomized to one of the four coded products representing the two formulas. Randomisation was performed centrally, at Harokopio University, based on computer-generated schemes. For each paediatrician a distinct randomisation table was created to ensure that infants recruited within one site will be accurately randomized across treatments.

All study personnel, including the Principal Investigator (PI), the paediatricians, the research associates and the Sponsor's Project Manager were blinded to the study formulas. In addition, all subjects (i.e. their parents) were blinded to the study formulas. All formulas were provided in similar blank tins of 400 g each that carried the description "not for commercial use". Four different codes were printed on the bottom of the tins in order to ensure blindness of the study:

- A85757 and A85758 for the control formula
- A85759 and A85760 for the test formula

Only Sponsor's specific employees had access to the recipe each code corresponded to.

The nutritional composition of the two study products can be seen in Table 2.2 below. The composition of the control formula on macro-nutrients was similar to the composition of the test formula apart from the protein fraction and both formulas complied with the compositional requirements laid down in Directive 2016/127/EC (European Commission, 2015).

Table 2-2. Nutritional composition of the SHIFT study formulas (per 100 ml).

	Test product	**Control product**
Energy (kcal)	66	66
Intact protein (g)		1.4
Protein hydrolysate (g)	1.6	
Fat (g)	3.5	3.5
DHA (mg)	6.9	6.9
AA (mg)	6.9	6.9
Carbohydrates	7.0	7.0
GOS (g)	0.3	0.4
Ca (mg)	50	56
P (mg)	30	31
Na (mg)	20	23
Fe (mg)	0.78	0.77
Cu (μg)	50	47
K (mg)	65	79
Mg (mg)	6	6.4
Mn (μg)	17	16
Zn (mg)	0.60	0.60
Cl (mg)	42	47
I (μg)	10	9
Se (μg)	1.7	2.5
Vitamin A (μg-RE)	70	74
Vitamin D (μg)	1.2	1.1
Vitamin E (mg)	1.3	1.7
Vitamin K (μg)	5.1	6.2
Vitamin B1 (μg)	59	57
Vitamin B2 (μg)	91	78
Niacin mg	0.47	0.49

Vitamin B6 (μg)	39	58
Vitamin B12 (μg)	0.16	0.16
Folic acid (μg)	10	11
Pantothenic acid (μg)	0.33	0.40
Biotin (μg)	1.4	1.7
Vitamin C (mg)	9.1	11
Nucleotides (mg)	3.25	3.25
Taurine (mg)	6	7.3
Choline (mg)	14	21
Inositol (mg)	3.9	4.4
Carnitine (mg)	1.7	1.6

2.1.2.4 Study procedures

Once the informed consent form was obtained, baseline anthropometric measurements (weight, length, and head circumference) were performed by the paediatrician, while family demographic information, perinatal, and birth characteristics of study participants were also collected. Three follow-up visits were performed thereafter, at the following time-points: Baseline +30, +60, and +90 days, with an allowed deviation of +/-2 days (Figure 2.3). Formula intake was assessed using a paper diary, which was completed by the parent/legal guardian on seven consecutive days before the visit to the paediatrician. At each visit, the formula intake diary was collected and a clinical examination to obtain anthropometric measurements was performed by the paediatrician. AEs, SAEs, and medication use were recorded during the follow-up visits and monitored by an independent paediatrician.

Furthermore, the IGSQ was used to assess infant's overall gut comfort and minor digestive issues (i.e. vomits/regurgitation, colic, constipation, diarrhoea and crying episodes). The questionnaire was filled in by the research assistant during an interview with the parents at each scheduled home visit. In addition, the AISS was used to assess the consistency (four categories: watery, soft, formed and hard), amount/volume (smear to more than 50 % of the nappy's surface) and colour (six categories) of stools. The AISS was filled in by parents at home whenever their infant defecated in the three days before the scheduled visit by the research assistant.

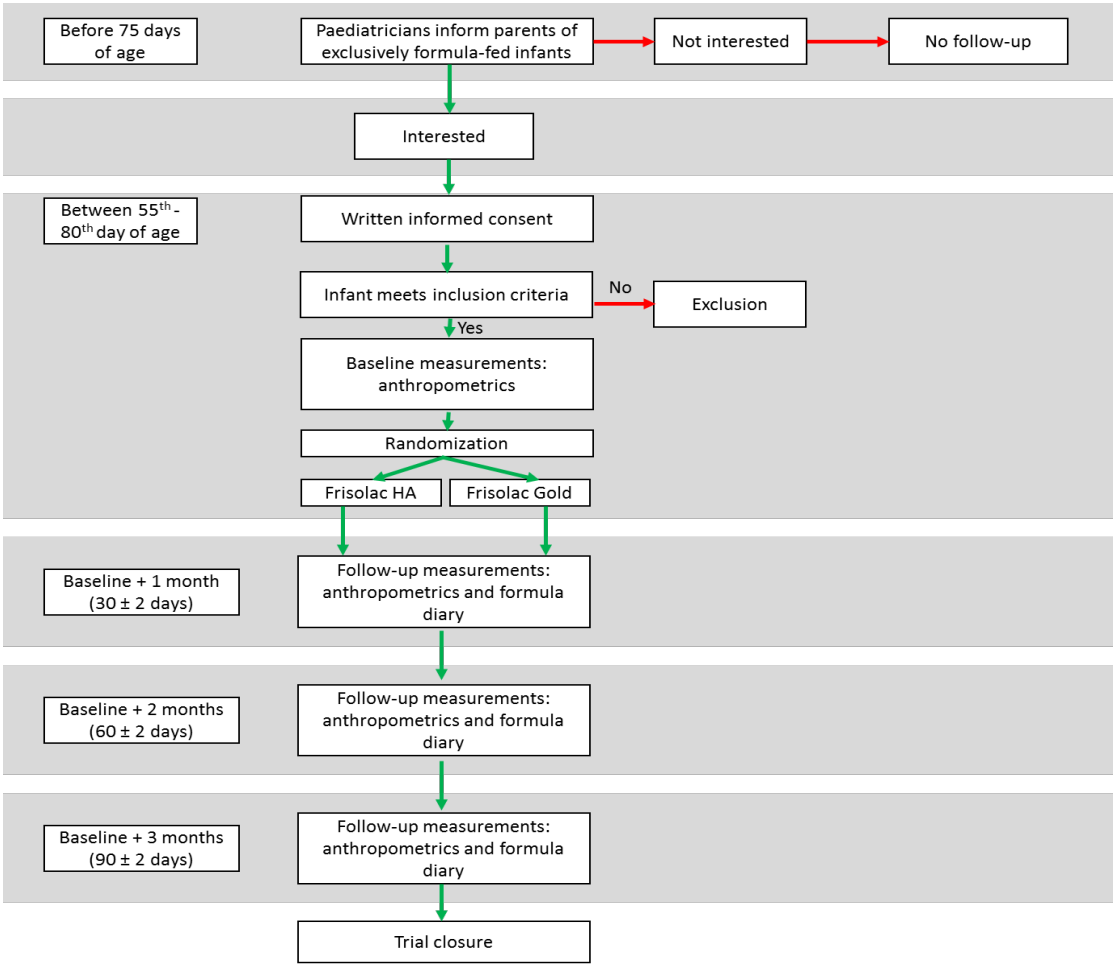

Figure 2-3. SHIFT Study flowchart.

2.1.2.5 Outcome Measures

For the assessment of the primary outcome (weight gain during the three-month intervention in g/day), infants' weight at baseline and at the 3rd follow-up visit was used as outcome variable.

In order to evaluate the secondary outcomes, the following indices were measured:

- weight (g), length (cm) and head circumference (cm) at baseline and at each follow-up visit,
- weight gain (g/day), recumbent length gain (cm/day) and head circumference gain (cm/day) from baseline to each one of the follow-up visits,
- Body mass index (BMI) (kg/m^2) from baseline to each one of the follow-up visits,

- weight-for-length, weight-for-age, length-for-age, BMI-for-age and head circumference-for-age Z-scores, generated using World Health Organisation (WHO) Anthro Survey Analyser, version 3.2.2, 2011(WHO, 2010), at each follow-up visit, as well as from baseline to each one of the follow-up visits.

For the tertiary outcomes, gastrointestinal comfort was measured with two questionnaires: the IGSQ and the AISS. Regarding the AISS, a mean stool consistency score was calculated out of the mean daily score from the three days. Similarly, a mean score for amount and colour of stools was calculated. Regarding the IGSQ, a continuous score per infant per visit was calculated ranging from 13 through 65.

2.2 ETHICS STATEMENT

Both “Little Panda” study and “SHIFT” study were performed in accordance with the guidelines of the Declaration of Helsinki and the International Conference on Harmonization (ICH) guidelines on Good Clinical Practice (GCP). Both study protocols, information letters to the parents/legal guardians, and written informed consent forms were approved by Harokopio University’s Ethics Committee. Furthermore, both studies were registered in the Netherlands Trial Registry [identifier Little Panda: NL6702 (NTR6872); identifier SHIFT: NL7378 (NTR7586)].

3. SAMPLE SIZE AND STATISTICAL ANALYSIS

3.1 LITTLE PANDA STUDY

3.1.1 Sample size calculation

Power calculation and sample size estimation for Little Panda study was based on the one available cross-over clinical trial by Carnielli et al. (Carnielli et al., 1995) that examined whether higher levels of PA in the sn-2 position of IF improves fat, FAs, and calcium absorption in infants. Based on the results reported for the concentration of PA in the faeces and after adjusting for dose and duration, the statistical power calculation revealed that a sample size of 10 infants (5 per treatment arm) per cross-over study is considered adequate to provide a significant difference of 25.2 mg/g wet faeces (SD of the difference 13.9) during the 4-week duration of the cross-over design of Little Panda study (power 80 %, level of significance 5 %). However, considering the higher pairwise difference in the SN-2-palmitate content of the two formulas used in the study by Carnielli et al., as well as that longer duration intervention studies examining similar outcomes have indicated larger sample sizes, a total sample size of 8 infants with complete data should be included per treatment arm. Taking an additional drop-out rate of 30 % into consideration, a sample size of 11 infants per treatment arm per cross-over study had to be recruited for the purpose of the Little Panda study.

3.1.2 Statistical analysis

A detailed Statistical Analysis Plan (SAP) was developed after the completion of data collection and prior to the database lock. All completed subjects that participated into the study were included in all the analyses (demographic and baseline characteristics, efficacy, and safety). Safety analyses were performed on all enrolled subjects who participated in the study and received either of the study formulas. Data analyses were performed with the study groups coded and the code was not broken until all analyses had been completed.

The two cross-over studies were analysed independently from each other. The primary outcomes were excretion of PA and PA soaps in stool. A hierarchical approach was taken when interpreting the results, with PA in stool tested first for statistical significance, followed by PA soaps in stool. Therefore, no further adjustments for multiplicity were conducted on the p-values. Analysis of variance (ANOVA) appropriate for a 2×2 cross-over design was used to assess mean differences

in stool PA and PA soap composition. When the normality assumption was be met, variables were log-transformed, or Wilcoxon signed-rank test was applied. The statistical model included treatment, sequence and period as fixed effects, and subject (sequence) and residual error term as random effects.

The secondary outcomes were calcium absorption and stool consistency (using AISS). The same ANOVA approach was used for calcium excretion and stool consistency analysis. Milk intake comparisons between the formula groups were done using Mann-Whitney Utest. All statistical tests were two-sided and performed with $\alpha = 0.05$.

Additional exploratory analyses were performed on total FA, total FA soaps, FA and FA soaps (ANOVA as with primary outcomes).

3.2 SHIFT STUDY

3.2.1 Sample size calculation

The sample size calculation was based on a non-inferiority test, using a one-sided, two sample t-test for the comparison of change in weight gain at three months of age between treatment groups, at a 2.5 % significance level and a power of 80 %. For the margin of non-inferiority, a weight gain of -3 g/day was used, which is considered to be nutritionally relevant according to the U.S. Food and Drug Administration (FDA) (American Academy of Pediatrics Task Force, 2015). We assumed a standard deviation of 6.1 g/day for weight gain (Puccio et al., 2017). The randomization ratio between the test formula and control formula was 1:1.

The null hypothesis was that the weight gain in the test group was at least 3 g/day less than in the control group. The alternative hypothesis of non-inferiority was that the difference in weight gain between the treatment groups (test minus control) was smaller than -3 g/day. The null hypothesis of inferiority of the test formula to the control formula will be rejected at the 2.5 % significance level if the lower bound of the 95 % confidence interval of the mean difference in weight gain between treatment groups (test minus control group) is above the specified non-inferiority margin of -3 g/day.

The sample size required for achieving a power of 80 % of the primary outcome is 66 infants per treatment group in the per-protocol analysis at three months of age. Assuming that 30 % of

infants from the enrolled analysis set will be excluded for the per-protocol analysis, a minimum of 95 enrolled infants per treatment group was required.

3.2.2 Statistical analysis

A detailed SAP was developed after the completion of data collection and prior to the database lock. Data analyses were performed with the study groups coded and the code was not broken until all analyses had been completed.

Three data analysis sets were defined and used for final analysis:

- The intention-to-treat (ITT) data analysis set which included all infants randomized to the study formulas.

- The per protocol (PP) data analysis sets which included all infants of the ITT data analysis set, except in case of protocol deviations.

- The safety data analysis set (all subjects treated: AST). This data analysis set included all infants of the ITT data analysis set minus the ones who did not consume any formula at all.

Analysis of data using the ITT analysis set considered allocation of infants to formula groups as randomized. Analysis of data using the PP and the AST analysis sets considered allocation of infants to study formula received.

For analysis of the primary endpoint, a one-sided statistical significance level of 2.5 % was used, while for the secondary endpoints, a two-sided statistical significance level of 5 % was used. No correction for multiplicity was done because there was only one primary parameter.

The primary endpoint (weight gain during the three-month intervention in g/day) was analysed using an analysis of covariance (ANCOVA) model, with the study formula as a fixed factor and adjustments for multiple covariates, including baseline weight, sex, antibiotic use, birth weight, maternal pre-pregnancy BMI, father's current BMI, and average formula intake. The primary endpoint analyses were carried out in both the ITT and PP analysis sets.

The secondary endpoint analyses were also carried out in both the ITT and the PP analysis sets and were analysed using a mixed models repeated measures (MMRM) analysis, with the study formula and visit as fixed factors, adjusting for several covariates (as in primary outcome) and their interactions.

4. RESULTS

The results of this Doctoral Thesis are presented as research manuscripts (under review/accepted/published). The published manuscripts can be found in the Appendix A (page 125).

MANUSCRIPT 1: Effect of milk fat-based infant formulae on stool fatty acid soaps and calcium excretion in healthy term infants: two double-blind randomised cross-over trials.

Background

Human milk (HM) represents optimum nutrition for full-term babies throughout infancy and is designed to meet the needs of the growing infant in the first months after birth (Andreas et al., 2015). Triacylglycerols (TAGs) in HM provide approximately 50% of the energy as well as essential fatty acids (FAs) important for the overall development of the infant (Delplanque et al., 2015; Koletzko et al., 2001; Miles and Calder, 2017). Palmitic acid (PA), one of the major saturated fatty acids in HM (representing approximately 20–25% of total FAs), is predominantly esterified at the SN-2 position of TAGs (i.e. SN-2-palmitate) in HM (Andreas et al., 2015; Koletzko et al., 2001; Marie Straarup et al., 2006). Studies over the last two to three decades have provided increasing evidence that the SN-2-predominant positioning of PA in HM TAGs promotes the absorption of both PA and calcium in term and preterm infants (Bar-Yoseph et al., 2016; Miles and Calder, 2017; Nowacki et al., 2014; Petit et al., 2017).

The majority of infant formulas (IF) use a blend of vegetable oils as a source of fat. Compared to HM fat, in which 70–88% of the PA is esterified at the SN-2 position, commonly used vegetable oils have lower percentage of PA in the SN-2 position of TAGs (10–20%) (Marie Straarup et al., 2006). Therefore, vegetable fat (VF) blends consist of TAGs with PA predominantly bound to the SN-1 and SN-3 positions (Havlicekova et al., 2016; Marie Straarup et al., 2006). During digestion, PA at the SN-1,3 positions is released as free PA. In the alkaline environment of the small intestinal lumen, free PA interacts readily with cations (e.g. calcium) to form insoluble soaps (Innis, 2011; Lindquist and Hernell, 2010) that are associated with hard stools, gut discomfort and decreased absorption of PA and minerals by the infant (Innis, 2011; Petit et al., 2017; Quinlan et al., 1995). Increasing the ratio of SN-2 to SN-1 and SN-3 palmitate in IF could ensure higher

absorption of fat and minerals (calcium), as well as lead to reduced formation of insoluble soaps, thereby, minimizing gut discomfort.

Synthetic structured TAGs have been developed with higher proportion of PA in the SN-2 position (ranging from 35.9–74%) and lower levels of PA at the SN-1 and SN-3 positions. Favourable effects of IF containing such synthetic TAGs on FA, calcium absorption and stool consistency have been reported in healthy infants by several studies (Bar-Yoseph et al., 2016; Béghin et al., 2019; Carnielli et al., 1996, 1995; Kennedy et al., 1999; López-López et al., 2001; Lucas et al., 1997; Nowacki et al., 2014; Yao et al., 2014).

Bovine milk fat (MF) is naturally higher in SN-2-palmitate than VFs, with a level of approximately 40% (Havlicekova et al., 2016; Innis, 2011; Petit et al., 2017) and a higher ratio of SN-2 vs SN-1,3 palmitate. Furthermore, MF shows comparable TAG structures to those in HM fat (Petit et al., 2017). Therefore, using MF in the development of IF may enable mimicking the composition and structure of HM fat, potentially leading to a higher absorption of PA and calcium, less soap formation and softer stools in comparison to IF containing VF only.

This paper reports on two studies. Each study was a double-blind, cross-over, randomised, placebo-controlled comparing a MF-based formula against a standard VF formula. The primary objective of these studies was to evaluate the excretion of PA and PA soaps in stools of healthy term infants. We hypothesized that infants fed MF-based IF had lower PA and PA soaps in stool when compared to infants fed VF-based formula. In addition, the secondary outcomes of both studies were calcium excretion in stools, stool consistency scores and other FA and FA soaps in stools.

Methods

Study design and population

The present studies were two separate double-blind, cross-over, randomised, placebo-controlled trials, conducted in parallel with healthy, full-term, exclusively formula-fed (FF) infants (Figure 4.1). Sampling and recruitment were performed by paediatricians at 12 private paediatric clinics in two cities (Athens and Larissa) in Greece between December 2017 and July 2018. Infants were screened between their 9th–14th week of age on the following inclusion criteria: full-term, healthy (born at gestational age ≥ 37 weeks), exclusively FF infants, with appropriate for

gestational age birthweight. Exclusion criteria were: i) severe acquired or congenital diseases, mental or physical disorders, any symptoms of allergy (including cow's milk allergy); ii) Use of probiotics, antibiotics or other medication that treat or cause GI symptoms; iii) use of medication(s) known or suspected to affect fat digestion, absorption and/or metabolism, nutritional supplements, suppositories, medication that may suppress or neutralize gastric acid secretion and gut motility at the time of screening or at any time throughout the study period; iv) participation in another clinical trial; v) any type of mixed feeding (See Supplementary file 1 for full inclusion and exclusion criteria). Written informed consent was obtained from parents after explanation of the study procedures and prior to inclusion into the study. The study procedures were initiated immediately upon inclusion.

The protocol, information letter to the parents/caregivers and written informed consent form were approved by Harokopio University's Ethics Committee. The study was conducted in accordance with the guidelines of the Declaration of Helsinki and the International Conference on Harmonisation (ICH) guidelines on Good Clinical Practice (GCP) and was registered in the Netherlands Trial Registry (identifier: NTR6702).

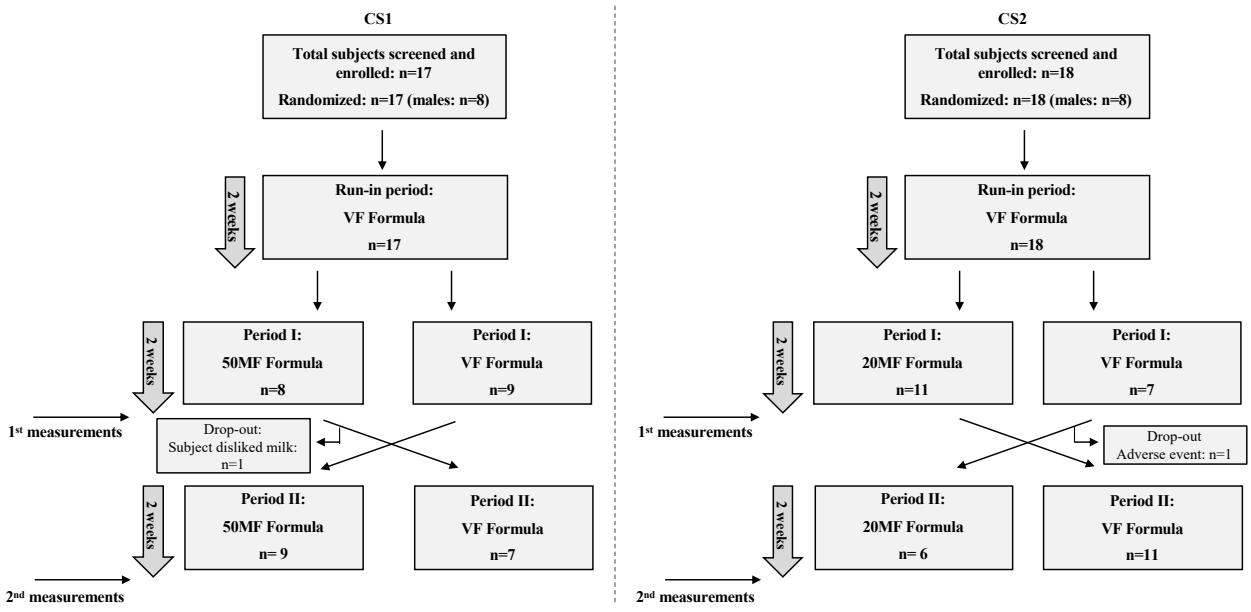

Figure 4-1. Study flowchart and subjects' disposition.

CS1: cross-over study 1; CS2: cross-over study 2. MF: milk fat; VF: vegetable fat; 50MF: 50% MF formula; 20MF: 20% MF formula

Study randomisation and formulas

Upon inclusion in the study, all infants were fed the 100% VF formula with 10.1% SN-2-palmitate levels (total PA 24.9%) for 2 weeks (run-in period) in order to minimize the potential effects of previous feedings. Infants were then allocated to one of the cross-over studies using block randomisation. In each of the studies infants were randomly assigned to receive either the VF formula or a MF-based formula: i) 50% MF + 50% VF (50MF) with 39% SN-2-palmitate levels (total PA 18.9%) in cross-over study 1 (CS1) and ii) 20% MF + 80% VF (20MF) with 19.7% SN-2-palmitate levels (total PA 26.1%) in cross-over study 2 (CS2). Randomisation into the two treatment arms per study was based on a computer-generated sequence. After 2 weeks (period I), infants were crossed over to receive the other formula for another 2 weeks (period II) in their respective CS1 and CS2 (Figure 4.1). The nutritional composition of the three study formulas was similar with the only difference being their FA profiles and percentage of SN-2-palmitate (Table 4.1). The procedures followed for the determination of SN-2-palmitate and total FA profile of study products can be found in Supplementary file 2. All powder properties were identical between the control and experimental formulas. All formulas were produced in the Netherlands by FrieslandCampina and were packaged in similar blank tins of 400 g each with a specific identification code at the bottom of the tins. The study formulas were labelled by the manufacturer using a single letter per formula group (A, B, C, D or E). The manufacturer retained the codes for the study formulas. All study personnel, including the Principal Investigator and the Sponsor's Project Manager as well as parents/caregivers were blinded to the formula's allocation. Sealed envelopes containing product codes were provided to the study site in the event of an emergency. The tin label included guidance for the parents on the daily volume of formula intake required by the infant, which depended upon age and weight.

Table 4-1. Composition of the study formulas.

	Formula		
Nutrient/ingredient	**50MF**	**20MF**	**VF**
Energy (kcal/100mL)	66	66	66
Intact protein (g/100mL)	1.4	1.4	1.4
Carbohydrates (g/100mL)	7.1	7.0	7.0
Galacto-oligosaccharides (g/100mL)	0.27	0.27	0.27
Fat (g/100mL)	3.5	3.5	3.5

Docosahexanoic acid (mg/100mL)	6.9	6.9	6.9
Arachidonic acid (mg/100mL)	8.3	8.3	6.9
Fatty acids; mol % of TAGs			
C12:0; Lauric acid	6.0	7.7	10.4
C14:0; Myristic acid	7.4	4.8	3.9
C16:0; Palmitic acid	18.9	26.1	24.9
C18:0; Stearic acid	5.2	4.4	3.4
C18:1; Oleic acid	36.9	42.2	39.0
C18:2; Linoleic acid	11.7	16.4	12.7
C18:3; a-Linolenic acid	1.5	1.6	1.8
C20:0; Arachidic acid	0.2	0.3	0.3
% C16:0 in sn-2 position	39	19.7	10.1
Calcium (mg/100mL)	53	55	56

MF: milk fat; VF: vegetable fat. 50MF: 50% MF formula; 20MF: 20% MF formula
To ensure double-blindness, all formulas were packaged in similar blank tins of 400 g each with different identification codes at the bottom of the tins. Formula labels provided preparation, storage and feeding instructions in both English and Greek.

Stool collection and analysis

Stool samples were collected at home by parents/caregivers for three consecutive days at the end of period I and period II for analysis of their FAs, FA soaps and calcium content. Each freshly passed stool was placed in a faecal tube collector (until 30 g was collected in total), kept in a ziplock amber plastic bag and then stored in the home freezer. At the end of each intervention period, the study personnel collected the stool samples from the homes and brought them to Harokopio University. The stool samples were stored in Harokopio University in a freezer at -80 °C until being transported in dry ice to Covance Laboratory, Madison, Wisconsin, USA for analysis. The analytical procedures followed in the laboratory are described in Supplementary file 2.

Formula consumption and stool characteristics.

Parents/caregivers were asked to record formula consumption using a three-day milk diary, where the timing, frequency as well as the exact amount/volume (in mL) of formula consumed were recorded during the same 3 days of each intervention period as stool collection. Additionally, the study personnel collected all formula tins to monitor compliance and formula consumption.

Stool characteristics assessment was performed by parents/ caregivers using the validated Amsterdam Infant Stool Scale (AISS) (Bekkali et al., 2009), which assesses the consistency, amount/volume and colour of stools. For assessment of consistency, each freshly passed stool during the three-day period was evaluated and ranked accordingly on a scale of one to four (watery = 1, soft = 2, formed = 3, hard =4) and a mean score was calculated.

Safety and anthropometric assessment

Adverse events (AEs) and serious adverse events (SAEs) were recorded throughout the study and monitored by an independent paediatrician. No code-break requests occurred for AEs or SAEs throughout the study and de-blinding did not need to take place. Anthropometric indices (weight and length) were also measured following standardized procedures at screening and at the end of the run-in period, period I and period II.

Statistical analysis

Sample size for both studies was determined based on the data from one available cross-over study by Carnielli et al. (Carnielli et al., 1995) on the concentration of PA in stools in infants fed control and high SN-2-palmitate formula, and adjusted for dose and duration. At least 16 infants per cross-over study were required to achieve a power of 80% ($\alpha = 0.05$) to detect a mean (SD) between-group difference of 25 (13.9) mg PA per /g of wet stool between VF control IF and MF-based IF. Assuming an expected 30% drop-out rate, 22 infants per cross-over study were required to achieve 16 evaluable infants per cross-over study. Data analyses were performed with the study groups coded; the code was not broken until all analyses had been completed.

The two cross-over studies were analysed independently from each other by 4Pharma Ltd. (Finland) using SAS® version 9.4 for Windows (SAS Institute Inc., Cary, NC, USA). The primary outcomes were excretion of PA and PA soaps in stool. A hierarchical approach was taken when interpreting the results, with PA in stool tested first for statistical significance, followed by PA soaps in stool. Therefore, no further adjustments for multiplicity were conducted on the p-values. ANOVA appropriate for a 2×2 cross-over design was used to assess mean differences in stool PA and PA soap composition. When the normality assumption was not met, variables were log-transformed or Wilcoxon signed-rank test was applied. The statistical model included treatment, sequence and period as fixed effects, and subject (sequence) and residual error term as random effects.

The secondary outcomes were calcium absorption and stool consistency (using AISS). The same ANOVA approach was used for calcium excretion and stool consistency analysis. Milk intake comparisons between the formula groups was done using Mann-Whitney Utest. All statistical tests were two-sided and performed with $\alpha = 0.05$.

Additional exploratory analyses were performed on total FA, total FA soaps, FA and FA soaps (ANOVA as with primary outcomes).

Results

Study population

From the total infants enrolled in CS1 and CS2 (n = 17 and n = 18, respectively), one infant dropped out of CS1 (subject disliked milk) and one from CS2 (subject had adverse event, not related to study product). The total number of infants that completed CS1 and CS2 was n = 16 and n = 17, respectively (Figure 4.1). It was decided to stop recruitment when each cross-over study had at least 16 infants completing the study. The overall drop-out rate was below 10% (2 subjects dropped out).

The baseline and family characteristics of the subjects are descriptively presented in Table 4.2. Weight at birth, gestational age as well as infants age and weight at inclusion were similar among the groups per cross-over study.

Table 4-2. Baseline infant & family characteristics.

	CS1		**CS2**	
	50MF - VF (n=7)	**VF – 50MF (n=9)**	**20MF - VF (n=11)**	**VF - 20MF (n=6)**
Gender, No. (%) male	3 (43)	5 (56)	6 (55)	2 (33)
Age at screening, mean (SD), days	103 (16)	92 (22)	95 (18)	96 (17)
Weight at screening, mean (SD), g	6368 (798)	5380 (1018)	5941 (1105)	5192 (722)
Mother's age, mean (SD), years	34 (7)	32 (5)	35 (8)	33 (4)
Mother's education level:				
No. (%) <12 years	2 (29)	3 (33)	5 (46)	1 (17)
No. (%) 12-14 years	2 (29)	2 (22)	1 (9)	3 (50)
No. (%) >14 years	3 (43)	4 (44)	5 (46)	2 (33)
Gestational age, mean (SD), weeks	39 (2)	38 (1)	39 (1)	38 (1)

Mode of delivery				
No. (%) caesarean section	4 (57)	7 (78)	6 (55)	5 (83)
Weight at birth, mean (SD), g	3259 (491)	2883 (391)	3143 (399)	2833 (318)

Data are descriptively summarized, given the cross-over design of the study.
CS1: cross-over study 1; CS2: cross-over study 2; SD: standard deviation; 50MF: 50% MF formula; 20MF: 20% MF formula; MF: milk fat; VF: vegetable fat.

Formula consumption and anthropometric data

The average weekly milk intake or the subjects' weight and length measurements at the end of the two-week intervention periods did not differ between the MF and VF groups in either of the cross-over studies (Supplementary file 3).

Stool fatty acids

The faecal concentrations of the major FAs are reported in Table 4.3. No significant difference was noted in the PA in stool between the MF-based IF and VF formula in both, CS1 and CS2. Similarly, no difference was observed for the total free FAs between the MF-based IF and VF formula.

The MF-based IF group in both cross-over studies had lower Lauric acid (C12:0) concentrations (CS1: $p < 0.0001$; CS2: $p = 0.004$) than VF group. In contrast, the opposite was observed for Myristic (C14:0) and Stearic (C18:0) in the MF-based IF groups ($p < 0.05$) in both, CS1 and CS2. The 50MF group (CS1) also had higher level of Gamma Linolenic acid than the VF group ($p < 0.05$).

In addition, Table 4.3 presents the faecal concentrations of the major FAs as the % of each FA within total free FAs lost in one g of dry stool. In CS1, the 50MF group had a decreased % of PA ($p = 0.0003$) and Lauric acid ($p < 0.0001$), and increased % of Myristic and Stearic acids ($p < 0.0001$) compared to the VF group. In CS2, no differences were observed in the % of PA, however, a decreased % of Lauric acid was observed in the 20MF group compared to the VF group ($p = 0.0002$).

Table 4-3. Stool fatty acids and calcium composition (mg/g stool dry weight).

CS1			CS2		
	50MF (N=16)	**VF (N=16)**		**20MF (N=17)**	**VF (N=17)**
Free Fatty Acids			**Free Fatty Acids**		
Palmitic acid (C16:0)†	4.4 (3.4 – 10.3)	5.7 (4.4 – 9.1)	Palmitic acid (C16:0)*	5.9 (3.8 – 13.4)	4.9 (3.8 – 7.3)
Lauric acid (C12:0)†	0.50 (0.28 – 0.78)[a]	1.38 (1.11 – 1.99)	Lauric acid (C12:0)‡	1.30 (0.72)[b]	1.59 (0.840
Myristic acid (C14:0)‡	1.35 (0.70)[b]	1.00 (0.59)	Myristic acid (C14:0)*	0.98 (0.66 – 1.59)[b]	0.79 (0.64 – 1.00)
Stearic acid (C18:0)†	1.83 (1.25 – 4.37)[b]	1.25 (0.93 – 1.84)	Stearic acid (C18:0)*	1.40 (0.92 – 2.94)[b]	0.99 (0.83 – 1.48)
Oleic acid (C18:1 n-9)†	4.80 (3.32 – 7.84)	5.01 (3.91 – 8.30)	Oleic acid (C18:1 n-9)*	6.65 (4.09 – 8.29)	5.70 (4.65 – 7.43)
Linoleic acid (C18:2)†	0.73 (0.46 – 1.36)	0.84 (0.45 – 1.46)	Linoleic acid (C18:2)†	0.93 (0.72 – 1.95)	0.88 (0.84 – 1.37)
Gamma Linolenic acid (C18:3 n-6)‡	0.08 (0.02)[b]	0.07 (0.02)	Gamma Linolenic acid (C18:3 n-6)‡	0.09 (0.04)	0.08 (0.02)
Alpha Linolenic acid (C18:3 n-3)*	0.07 (0.07 – 0.10)	0.07 (0.06 – 0.11)	Alpha Linolenic acid (C18:3 n-3)†	0.09 (0.07 – 0.19)	0.09 (0.08 – 0.15)
Arachidic acid	0.10 (0.07 – 0.18)	0.10 (0.09 – 0.17)	Arachidic acid	0.09 (0.07 – 0.17)	0.09 (0.08 – 0.12)

(C20:0)[†]			(C20:0)[*]		
Total FAs[‡]	22.37 (11.43)	23.16 (12.84)	Total FAs[*]	18.6 (15.7 – 32.7)	19.4 (15.3 – 22.3)
Fatty Acid Soaps			**Fatty Acid Soaps**		
Palmitic soap (C16:0)[‡]	111.28 (18.33)[a]	220.25 (29.35)	Palmitic soap (C16:0)[‡]	216.24 (25.16)[b]	233.94 (35.12)
Lauric soap (C12:0)[†]	1.76 (1.50 – 2.27)[a]	6.83 (5.74 – 7.67)	Lauric soap (C12:0)[‡]	4.38 (1.27)[a]	7.34 (1.88)
Myristic soap (C14:0)[‡]	10.82 (2.09)	11.24 (1.37)	Myristic soap (C14:0)[*]	11.90 (10.90 – 13.20)	12.20 (11.10 – 12.70)
Stearic soap (C18:0)[‡]	50.92 (7.81)[a]	31.21 (4.78)	Stearic soap (C18:0)[*]	39.50 (38.40 – 46.40)[b]	36.40 (31.20 – 37.60)
Oleic soap (C18:1 n-9)[†]	10.02 (7.05 – 14.05)	8.72 (7.61 – 12.65)	Oleic soap (C18:1 n-9)[‡]	10.10 (6.11)[b]	11.63 (7.29)
Linoleic soap (C18:2)[†]	1.11 (0.70 – 1.42)	1.13 (0.92 – 1.47)	Linoleic soap (C18:2)[‡]	1.21 (0.70)[b]	1.57 (0.98)
Total FA soaps[‡]	201.63 (34.79)[a]	290.19 (42.81)	Total FA soaps[‡]	296.59 (31.29)[b]	311.18 (39.75)
Calcium			**Calcium**		
Stool calcium[‡]	46.40 (5.27)[b]	49.88 (4.77)	Stool calcium[‡]	46.20 (4.26)[b]	50.47 (6.71)

[‡] Analysis of variance for variable in original scale of measurement. Data are presented as mean (SD).

[†] Analysis of variance for log-transformed variable. Data are presented as median (IQR).

[*]Non-parametric analysis (Wilcoxon Signed Rank). Data are presented as median (IQR).

P-values indicated by a, p<0.0001; b, p<0.05 are not eligible for statistical significance according to pre-defined hierarchy.

CS1: cross-over study 1; CS2: cross-over study 2; 50MF: 50% MF formula; 20MF: 20% MF formula; MF: milk fat; VF: vegetable fat; SD: standard deviation; IQR: inter-quartile range.

Stool fatty acid soaps

The MF-based IF groups in both CS1 and CS2 had a lower concentration of total FA soaps in stool than the VF group (Table 4.3; CS1: $p < 0.0001$; CS2: $p = 0.0077$). In CS1, the 50MF group had a lower concentration of PA soaps in stool compared to the VF group ($p < 0.0001$). Similar results were also noted in CS2, with lower PA soaps in the 20MF group ($p = 0.0023$). In CS1, Lauric acid (C12:0) soap concentrations were lower ($p < 0.0001$), whilst Stearic acid (C18:0) soap concentration was increased in the 50MF group compared to the VF group ($p < 0.0001$). In CS2, a decrease in Lauric (C12:0), Oleic (C18:1) and Linoleic acid (C18:2) soap concentrations were observed in the 20MF group compared to the VF group ($p < 0.05$). Stearic acid (C18:0) soap concentration, however, was increased ($p = 0.0021$) (Table 4.3).

In addition, Table 4.4 presents the faecal concentrations of the major FA soaps as the % of each FA soap within total FA soaps lost in one g of dry stool. In CS1 and CS2 both, 50MF and 20MF groups had decreased % of PA soaps compared to the VF group (CS1: $p < 0.0001$; CS2: $p = 0.0032$). In CS1, similar results were observed for the % of Lauric acid (C12:0) soaps ($p < 0.0001$), while the opposite was observed for Myristic (C14:0), Stearic (C18:0) and Oleic acid (C18:1) soaps ($p < 0.0001$). In CS2, a decrease was observed for the % of Lauric (C12:0) and Linoleic acid (C18:2) soaps ($p < 0.0001$ and $p = 0.0059$, respectively), while the opposite was observed for Myristic (C14:0) and Stearic acid (C18:0) soaps ($p = 0.0058$ and $p = 0.0026$, respectively).

Table 4-4. Percentages of individual FAs and FA soaps within total free FAs and total FA soaps, respectively.

CS1			CS2		
	50MF (N=16)	**VF (N=16)**		**20MF (N=17)**	**VF (N=17)**
% Individual Fatty Acids within Total Free FAs			**% Individual Fatty Acids within Total Free FAs**		
% Palmitic acid (C16:0)‡	28.79 (8.41)[b]	35.88 (10.46)	% Palmitic acid (C16:0)*	31.2 (23.0 – 36.0)	29.3 (24.3 – 36.0)
% Lauric acid (C12:0)‡	2.39 (0.73)[a]	7.05 (1.94)	% Lauric acid (C12:0)‡	4.99 (1.78)[b]	7.28 (2.25)
% Myristic acid (C14:0)‡	6.06 (1.01)[a]	4.26 (0.56)	% Myristic acid (C14:0)‡	4.44 (0.92)	4.08 (0.62)
% Stearic acid (C18:0)‡	11.57 (3.96)[a]	7.20 (1.94)	% Stearic acid (C18:0)*	7.43 (5.64 – 8.23)	5.73 (5.45 – 6.76)
% Oleic acid (C18:1 n-9)‡	29.74 (10.25)	28.23 (9.07)	% Oleic acid (C18:1 n-9)‡	31.11 (7.95)	28.51 (7.71)
% Linoleic acid (C18:2)‡	4.66 (2.63)	4.39 (1.84)	% Linoleic acid (C18:2)‡	5.68 (2.47)	5.79 (2.54)
% Gamma Linolenic acid (C18:3 n-6)†	0.37 (0.30 – 0.57)	0.35 (0.27 – 0.41)	% Gamma Linolenic acid (C18:3 n-6)†	0.32 (0.26 – 0.47)	0.35 (0.32 – 0.47)
% Alpha Linolenic acid (C18:3 n-3)‡	0.50 (0.215)	0.44 (0.18)	% Alpha Linolenic acid (C18:3 n-3)‡	0.52 (0.23)	0.57 (0.27)

% Arachidic acid (C20:0)‡	0.58 (0.15)	0.59 (0.19)	% Arachidic acid (C20:0)†	0.52 (0.46 – 0.60)	0.54 (0.46 – 0.64)
% Fatty Acid Soaps within Total FA Soaps			**% Fatty Acid Soaps within Total FA Soaps**		
% Palmitic soap (C16:0)*	54.4 (54.1 – 57.3)[a]	76.2 (75.6 – 77.6)	% Palmitic soap (C16:0)*	72.7 (71.5 – 74.7)[b]	76.6 (74.0 – 77.3)
% Lauric soap (C12:0)‡	0.93 (0.25)[a]	2.46 (0.38)	% Lauric soap (C12:0)‡	1.48 (0.42)[a]	2.36 (0.53)
% Myristic soap (C14:0)‡	5.36 (0.35)[a]	3.89 (0.15)	% Myristic soap (C14:0)*	4.05 (3.88 – 4.12)[b]	3.93 (3.71 – 3.96)
% Stearic soap (C18:0)†	25.52 (23.95 – 26.48)[a]	10.73 (10.34 – 11.01)	% Stearic soap (C18:0)*	14.04 (13.10 – 14.87)[b]	11.24 (10.31 – 11.78)
% Oleic soap (C18:1 n-9)‡	5.45 (2.17)[b]	3.94 (1.88)	% Oleic soap (C18:1 n-9)‡	3.38 (1.77)	3.72 (2.14)
% Linoleic soap (C18:2)‡	0.62 (0.29)	0.50 (0.26)	% Linoleic soap (C18:2)‡	0.40 (0.21)[b]	0.51 (0.29)

‡ Analysis of variance for variable in original scale of measurement. Data are presented as mean (SD).

† Analysis of variance for log-transformed variable. Data are presented as median (IQR).

**Non-parametric analysis (Wilcoxon Signed Rank). Data are presented as median (IQR).*

P-values indicated by a, p<0.0001; b, p<0.05 are not eligible for statistical significance according to pre-defined hierarchy.

CS1: cross-over study 1; CS2: cross-over study 2; 50MF: 50% MF formula; 20MF: 20% MF formula; MF: milk fat; VF: vegetable fat; SD: standard deviation.

Stool calcium

The mean calcium concentration in stools was lower in both 50MF and 20MF groups compared to their respective VF group (CS1: p = 0.0041; CS2: p = 0.0067; Table 4.3).

Stool consistency

The mean stool consistency is presented in Figure 4.2. In CS1, the mean stool consistency score was decreased in 50MF group compared to the VF group (p = 0.0032). Parents/caregivers of infants in the 50MF group reported watery and soft stools, while the VF group reported only soft stools. The mean stool consistency score in CS2 did not differ between the 20MF and VF groups, and was classified as soft.

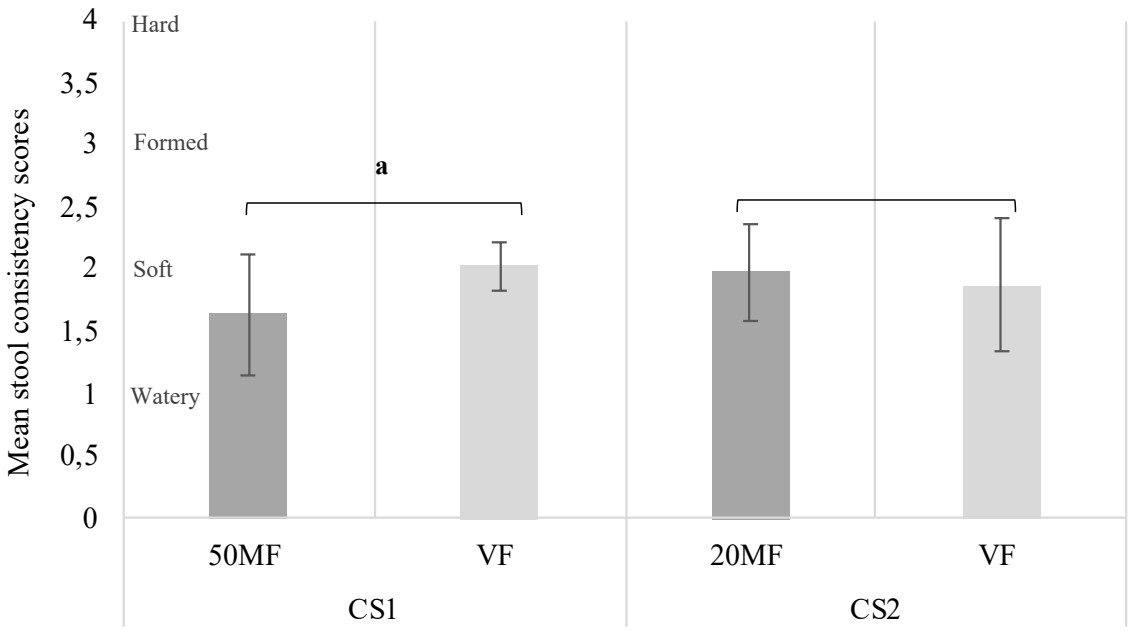

Figure 4-2. Stool consistency scores according to feeding group.

Individual stool consistency scores were determined using the Amsterdam Infant Stool Scale (AISS) (categorization: 1 = watery, 2 = soft, 3 = formed, and 4 = hard). Comparisons between the formula groups were conducted using analysis of variance. Values are means (± standard deviation).

CS1: cross-over study 1; CS2: cross-over study 2. MF: milk fat; VF: vegetable fat. Significant difference between the 50MF and the VF group: p=0.0032.

Discussion

To our knowledge, this is the first study assessing the effect of IF with bovine MF on stool FAs, FA soaps and calcium excretion in healthy term infants. Although, current studies did not show a

significant difference on PA in stool as initial primary outcome measure, an interesting observation is that both, 50MF and 20MF formulas did demonstrate favourable effects on PA soaps in stool and other secondary outcomes, e.g. calcium excretion and total FA soaps in stools, compared to the VF formula. This underlines the importance of further exploration of bovine MF application in IF. Additionally, various FA showed different trends in FA soap concentrations with increase of MF content in the IF. As the IF in the current study differed in their overall FA profile, it is likely that this contributed to the observed FA trends and not just their distribution over SN-2 and SN-1,3 positions.

Interestingly, 50MF formula with high SN-2-palmitate levels favourably affected infants' stool consistency scores. These findings are in line with published literature, although the reported studies had different study designs, age groups of infants and/or duration of interventions (Bar-Yoseph et al., 2016; Carnielli et al., 1996, 1995; Kennedy et al., 1999; Lucas et al., 1997; Nowacki et al., 2014; Yao et al., 2014). Most of these studies have tested IF with synthetic TAGs at various proportions of SN-2-palmitate, in contrast to the current MF-based formulas.

All previous studies consistently report that a higher SN-2-palmitate content in IF results in improved PA and FAs absorption (Carnielli et al., 1996, 1995; Lucas et al., 1997) or lower faecal excretion, either as free PA and free FAs (Bar-Yoseph et al., 2016; Carnielli et al., 1995) or as PA soaps and FA soaps in the faeces (Bar-Yoseph et al., 2016; Kennedy et al., 1999; Nowacki et al., 2014; Yao et al., 2014). No differences were observed between the current test groups and their respective control group on the absolute PA concentrations in the faeces, only the proportion of PA within total FAs excreted in the faeces was lower in the 50MF group compared to the VF group. However, infants fed with both MF-based formulas, despite lower SN-2-palmitate levels than reported in literature for synthetic TAGs (Bar-Yoseph et al., 2016; Carnielli et al., 1996, 1995; Kennedy et al., 1999; Lucas et al., 1997; Nowacki et al., 2014; Yao et al., 2014), had lower amounts of PA soaps in their stools compared to the VF formula. Furthermore, infants fed 20MF also had lower faecal excretion of Oleic and Linoleic soaps compared to those receiving VF formula which can be speculated as an additional benefit of the increased SN-2-palmitate content using MF on the absorption of these essential FAs. This suggests that increasing the SN-2-palmitate content through the use of MF might have comparable favourable effects to synthetic TAGs even at a lower concentration.

Calcium excreted in the faeces was found to be lower in both MF groups compared to the VF group. This potentially suggests improved calcium absorption by the infants as reported by previous balance studies (Carnielli et al., 1996, 1995; Lucas et al., 1997). This finding is particularly relevant since the groups had comparable average IF intake and the calcium content in the formulas was similar. The potential health benefits of improved calcium availability on bone indices have been reported by two previous studies in healthy term infants which showed improved bone mass / bone strength / quality [as determined either by dual-energy x-ray absorptiometry (Kennedy et al., 1999) or by quantitative ultrasound measurements of bone speed of sound (Litmanovitz et al., 2013)] when a high (50 and 43%, respectively) SN-2-palmitate formula was used compared to a standard low (12 and 14%, respectively) SN-2-palmitate formula. A balance study to confirm whether the reduced faecal calcium excretion seen in this study correlates with improved calcium retention and absorption is warranted.

In this study we have used the AISS (Bekkali et al., 2009), which is considered a more appropriate tool for infants defecating in nappies (Ghanma et al., 2014) to assess stool consistency in SN-2-palmitate IF related studies. In general, FF infants have harder stools compared to breast-fed (BF) infants who typically have watery to soft stools (Quinlan et al., 1995). Differences in stool consistency have been mainly associated with the higher content of FA soaps in the faeces of FF infants compared to the BF ones (Quinlan et al., 1995). Results from previous studies, using different stool scales to assess the effect of IF with various SN-2-palmitate content on stool consistency, have been inconsistent. Two studies found that infants receiving a high (50 and 36%, respectively) SN-2-palmitate formula had softer, less-formed stools than infants in the low (12 and 12%, respectively) SN-2-palmitate formula groups (Kennedy et al., 1999; Yao et al., 2014). In contrast, the study by Nowacki et al. (Nowacki et al., 2014) showed no differences between the high (39%) and the low (13%) SN-2-palmitate groups. The study by Carnielli et al. (Carnielli et al., 1996) showed that infants fed the high (66%) SN-2-palmitate formula had a more favourable stool consistency score than the intermediate (39%) and low (13%) SN-2-palmitate formulas. Infants fed the intermediate formula had stool consistency scores between those of the high and the low SN-2-palmitate formulas. In the present study, infants consuming the 50MF formula had a mean score closer to the watery category [which is similar to the BF infants (Quinlan et al., 1995; Weaver et al., 1988)] and the infants consuming the VF formula had a mean score closer to the soft category, while no differences were observed for the 20MF formula vs. the VF group. The lack of difference between the 20MF formula and VF formula could be explained by the

absence of hard stool reports in any of the treatment groups, which might have limited the treatment effect induced by the 19.7% SN-2-palmitate levels in 20MF formula on stool consistency. Future studies including a reference group of BF infants may provide useful and relevant insights into stool consistency of infants.

Conclusions

In summary, while the MF-based IF did not affect the concentrations of PA in stool, our studies demonstrate that increasing SN-2-palmitate in IF using bovine MF results in lower palmitate soaps, total fatty acid soaps and calcium excretion in stools in healthy, term infants. Furthermore, a favourable effect on stool consistency is also noticed with the 50MF IF. The present studies suggest a role for application of bovine MF in IF. Further research to validate these favourable effects, taking into account stereospecificity of the triacylglycerol, and with the inclusion of a BF reference group is warranted.

Supplementary file 1. Inclusion and exclusion criteria

Inclusion criteria:

- Full-term, healthy infants (born at gestational age ≥37 weeks).
- "Appropriate for gestational age" birthweight (i.e. 10th centile ≤ Birth weight ≤ 90th centile).
- Age at enrolment: between 9th-14th week.
- Exclusively formula fed infants before and during the entire intervention period.
- Parents willing and agreeing to initiate complementary feeding after the end of endpoint measurements, i.e. after the completion of the 5th month of age.
- Parents willing to collect stools and fill in all study questionnaires and diaries during the entire intervention period.
- Written informed consent.

Exclusion criteria:

- Severe acquired or congenital diseases, mental or physical disorders, any symptoms of allergy (including cow's milk protein allergy; CMPA).
- Parents or siblings with documented CMPA allergy, diagnosed by a doctor.
- Use of probiotics, antibiotics or other medication that treat or cause GI symptoms and/or affect appetite at the time of screening or at any time throughout the study period (these infants will be considered as drop-outs).
- Use of medication(s) known or suspected to affect fat digestion, absorption and/or metabolism; nutritional supplements; suppositories; medication that may suppress or neutralize gastric acid secretion and gut mobility at the time of screening or at any time throughout the study period (these infants will be considered as drop-outs).
- Participation in another clinical trial.
- Any type of mixed feeding (i.e. combination of formula with breastfeeding in any proportion) and/or complementary feeding during the intervention.

Supplementary file 2. Biochemistry analysis

Prior to analysis of the stool samples, the samples were thawed, pooled, homogenized and lyophilized, and the % moisture was determined gravimetrically. The dried samples were extracted by solvent reflux to obtain the neutral lipids, including non-soaped free FA. The remaining samples were treated with acetic acid to release the soaped FA which were isolated by a second solvent reflux step. The free acids were isolated using solid phase extraction. The free acids were then converted to methyl esters using methanolic hydrochloric acid. The resulting FA methyl esters were analysed using a gas chromatograph equipped with a flame ionization detector and quantitated using external standards. Total FA soaps were calculated from the sum of all measured individual FA soaps. Both free FAs and soaped FAs were reported as mg/g dry weight stool in the acid form. Stool calcium content was determined by Inductively Coupled Plasma Emission Spectrometry using the AOAC International Official Methods of Analyses protocol[1].

Determination of SN-2-palmitate and total FA profile of Study products. The fat structure in IFT formulas was determined according to a SN-1/3 specific pancreatic lipase-based hydrolysis of TAG[2]. The 2-monoacylglycerols formed are isolated by thin layer chromatography and are subsequently methylated for gas chromatographic analysis (GC) and quantified in weight concentrations FA methyl esters (FAME). The latter is done by standard ISO methods[3,4]. For the conversion to molar FA concentrations, corrections are made for the FAME molecular weights.

References

[1]Pacquette LH, Thompson JJ, Malaviole I, et al. Minerals and Trace Elements in Milk, Milk Products, Infant Formula, and Adult/Paediatric Nutritional Formula, ICP-MS Method: Collaborative Study, AOAC Final Action 2015.06, ISO/DIS 21424, IDF 243. *J AOAC Int*. 2018;101(2):536-561.

[2]Luddy, F. E., Barford, R. A., Herb, S. F., Magidman, P., & Riemenschneider, R. W. (1964). Pancreatic lipase hydrolysis of triglycerides by a semimicro technique. Journal of the American Oil Chemists Society, 41(10), 693–696. https://doi.org/10.1007/BF02661412

[3]ISO15884 / IDF182. (2002). Milk fat – Preparation of fatty acid methyl esters.

[4]ISO15885 / IDF184. (2002). Milk fat – Determination of the fatty acid composition by gas-liquid chromatography.

Supplementary file 3.

Table S. 4-1. Formula consumption and anthropometric data at the end of the 2-week intervention periods.

	CS1			CS2		
	50MF (N=16)	**VF (N=16)**	**p-value**	**20 MF (N=17)**	**VF (N=18)**	**p-value**
Average weekly milk intake, mean (SD), mL	5707 (814)	6063 (1009)	0.28	5763 (1300)	6232 (1230)	0.3
Weight, mean (SD), g	6807.13 (918.45)	6706.25 (1089.85)	0.87	6566.35 (1047.99)	6697.78 (1139.31)	0.78
Length, mean (SD), cm	64.48 (2.74)	64.28 (3.71)	0.93	64.28 (2.87)	65.14 (3.18)	0.64

Comparisons between the formula groups were conducted using Mann-Whitney U-test.
CS1: cross-over study 1; CS2: cross-over study 2; 50MF: 50% MF formula; 20MF: 20% MF formula; MF: milk fat; VF: vegetable fat; SD: standard deviation.

MANUSCRIPT 2: A Partially Hydrolyzed Whey Infant Formula Supports Appropriate Growth: A Randomized Controlled Non-Inferiority Trial.

Introduction

Optimal feeding practices during early life are of utmost importance to support healthy growth and development in infants (Innis, 2014). Human milk represents the optimum nutrition throughout infancy and is associated with several short- and long-term benefits for both the child and the mother (Eidelman et al., 2012; Innis, 2014; Victora et al., 2016). However, when breastfeeding is not feasible, infant formulas (IF) are the best alternative.

Research has shown that infants who are formula-fed weigh more and have a higher risk of obesity later in life compared to breast-fed infants (Baird et al., 2005; Chomtho et al., 2008). Therefore, protein sources and IF processing technologies have been modified over the past years to optimize both the quality and the quantity of proteins in IF to better suit the nutritional requirements of infants and support more optimal growth. Protein hydrolysis, i.e., where proteins are digested into smaller fragments, peptides, or amino acids, is a frequent modification in IF, particularly those designed for special medical purposes (Yvan Vandenplas et al., 2014). Depending on the level of hydrolysis, hydrolysates can be classified as partially or extensively hydrolyzed proteins.

Hydrolysate-based formulas have been mainly developed for cow's milk protein allergy (CMPA) management, as IF containing extensively or partially hydrolyzed proteins are suggested to reduce the risk of developing allergic manifestations during the first four to six months of life (Alexander and Cabana, 2010; Von Berg et al., 2003), whilst extensively hydrolyzed formulas are successfully used in symptoms' management of existing CMPA (Høst et al., 1999; Koletzko et al., 2012). Furthermore, hydrolysate-based formulas are widely used for preterm infants, when breastfeeding is not available (Corvaglia et al., 2013; Koopman et al., 2009; Mihatsch et al., 2001), while some studies suggest potential benefits of partially hydrolyzed formulas (PHF) in the dietary management of common functional gastrointestinal symptoms such as fussiness, reflux, and colicky symptoms in formula-fed infants (Billeaud et al., 1990; Y. Vandenplas et al., 2014).

Despite the potential benefits of hydrolyzed protein formulas on CMPA prevention or gastrointestinal tolerance, it still needs to be evaluated whether growth indices remain comparable between infants fed standard intact protein formulas (IPF) and infants fed protein

hydrolysate-based IF. For this reason, new European Commission regulations (European Commission, 2015), applying to hydrolysate-based formulas from 2021 onwards, require that the safety and suitability of each specific hydrolysate-based IF is evaluated by clinical studies.

The primary objective of the current study was to evaluate the weight gain of healthy term infants consuming a whey-based PHF compared to a standard IPF over a period of three months. The secondary objective included evaluation of additional anthropometric indices at every timepoint over the period of three months.

Materials and Methods

Study Design and Population

This study was a double-blind, randomized controlled trial with two study arms: The test group consuming the PHF and the control group consuming the IPF. The study was conducted in healthy, full-term, exclusively formula-fed infants. Sampling and recruitment were performed by pediatricians in two cities (Athens and Larissa) in Greece between October 2018 (first subject in) and June 2019 (last subject in), while the overall study period ended in September 2019 (last subject out). Infants were enrolled between the 55th and 80th day of age during routine visits to the pediatricians. The inclusion criteria can be found in Supplementary file 1. Written informed consent was obtained from the parent/legal guardian of each infant before any study procedures were initiated.

The study protocol, information letter to the parents/legal guardians, and written informed consent form were approved by Harokopio University's Ethics Committee (approval code: 62/03-07-2018). The study was conducted in accordance with the guidelines of the Declaration of Helsinki and the International Conference on Harmonization (ICH) guidelines on Good Clinical Practice (GCP) and was registered in the Netherlands Trial Registry [identifier: NL7378 (NTR7586)].

Study Procedures and Formulas

Upon inclusion in the study, subjects were randomized to one of four coded products representing the two study formulas. Randomization was performed centrally, at Harokopio University, by a designated and trained research assistant based on computer-generated schemes. For each pediatrician a distinct randomization table was created to ensure that infants

recruited within one site would be appropriately randomized across treatments. Each time a pediatrician recruited an infant, the research assistant at Harokopio University was notified and she randomized the infant into one of the study groups. Next, she informed the pediatrician which coded formula the infant would be provided with, while also arranging delivery of the appropriate formula to the infant's house.

Formulas were provided for free to the participating families during the three-month study period and were used as the sole source of nutrition for the participating infants. Formula consumption was ad libitum but a feeding table in the "Parent Information Brochure" supported a correct consumption of the study products. The nutritional compositions of the IF used in this study are compliant to Commission Directive 2006/41/EC of 7 July 2006 amending Council Directive 91/414/EEC to include clothianidin and pethoxamid as active substances and are similar with regards to macro-nutrients, apart from the protein fraction (Table 4.5; for analytical composition of the two formulas see Supplementary file 2). Both IF were cow's milk based and were produced in the Netherlands by FrieslandCampina and packed in blank tins of 400 g each with a specific identification code at the bottom. All powder properties were identical between the test and control formulas. Parents/legal guardians, investigators, and study support staff were blinded to the formulas. Data analyses were performed with the study groups coded and the code was not broken until the database was locked.

Table 4-5. Composition of the study formulas (per 100 mL).

	Test Formula	**Control Formula**
Energy (kcal)	66	66
Intact protein (g)		1.4
Casein		0.57
Whey		0.85
Whey protein hydrolysate (g)	1.6	
Fat (g)	3.5	3.5
DHA (mg)	6.9	6.9
AA (mg)	6.9	6.9
Carbohydrates	7.0	7.0
GOS (g)	0.2	0.4
Ca (mg)	50	56
P (mg)	30	31

Na (mg)	20	23
Fe (mg)	0.78	0.77
Vitamin D (µg)	1.2	1.1

Test formula: Partially hydrolyzed whey infant formula; control formula: Intact protein formula; AA: Arachidonic acid; DHA: Docosahexaenoic acid; GOS: Galacto-oligosaccharides; Ca: Calcium; P: Phosphorus; Na: Sodium; Fe: Iron.

Once the informed consent form was obtained, baseline anthropometric measurements (weight, length, and head circumference) were performed by the pediatrician, while family demographic information, perinatal, and birth characteristics of study participants were also collected. Three follow-up visits were performed thereafter, at the following time-points: Baseline +30, +60, and +90 days, with an allowed deviation of +/-2 days. Formula intake was assessed using a paper diary, which was completed by the parent/legal guardian on seven consecutive days before the visit to the pediatrician. At each visit, the formula intake diary was collected and a clinical examination to obtain anthropometric measurements was performed by the pediatrician. Adverse events (AEs), serious adverse events (SAEs), and medication use were recorded during the follow-up visits and monitored by an independent pediatrician. No code-break requests occurred for AEs or SAEs throughout the study.

Primary and Secondary Outcome Measures

The primary outcome was weight gain (g/day) calculated as the difference in infant weight between the baseline and the 3rd follow-up visit, divided by the number of days between these visits. Secondary outcomes included other anthropometric indices assessed at each follow-up visit: Weight (g), length (cm), head circumference (cm), body mass index (BMI) (kg/m2), and their Z-scores (based on the World Health Organization (WHO) child growth standards (WHO Multicentre Growth Reference Study Group, 2006)). More details on the primary and secondary outcome measures can be found in Supplementary file 3.

Sample Size and Statistical Analysis

The sample size was determined according to guidelines from the American Academy of Pediatrics Task Force on Clinical Testing of Infant Formulas (American Academy of Pediatrics Task Force, 2015) and as described previously by Puccio et al. (Puccio et al., 2017). Specifically, the sample size calculation was based on a non-inferiority test, using a one-sided, two sample t-test

for the comparison of weight gain at three months of intervention between treatment groups. The PASS (version 15.0.4) software was used. For the margin of non-inferiority, a weight gain of -3 g/day was determined (American Academy of Pediatrics Task Force, 2015). Assuming a 2.5% significance level, a power of 80% and a standard deviation of 6.1 g/day (Puccio et al., 2017), 66 infants were needed in each formula group. The expected dropout rate was estimated to be 30%, mainly because of non-compliance to the required feeding strategy, thus enrolment of 95 infants per group was planned.

The null hypothesis was that the difference in weight gain between the test and control group would be higher than -3 g/day. The alternative hypothesis of non-inferiority was that the difference in weight gain between the two groups (test minus control) would be smaller than -3 g/day.

For analysis of the primary endpoint, a one-sided statistical significance level of $\alpha = 0.025$ was used, while for the secondary endpoints, a two-sided statistical significance level of $\alpha = 0.05$ was used. No correction for multiplicity was done, because there was only one primary parameter and missing data were not imputed.

The primary endpoint (weight gain during the three-month intervention in g/day) was analyzed using an analysis of covariance (ANCOVA) model, with the study formula as a fixed factor and adjustments for multiple covariates, including baseline weight, sex, antibiotic use, birth weight, maternal pre-pregnancy BMI, father's current BMI, and average formula intake. The adjusted mean and standard error (SE) of weight gain is reported. The primary endpoint analyses were carried out in both the intention-to-treat (ITT) and per-protocol (PP) analysis sets.

The secondary endpoint analyses were also carried out in both the ITT and the PP analysis sets and were analyzed using a mixed models repeated measures (MMRM) analysis, with the study formula and visit as fixed factors, adjusting for several covariates (see primary outcome) and their interactions.

Data were analyzed independently by the statistical company OCS Life Sciences. The statistical analyses were performed using the SAS software version 9.4 or higher (SAS Institute, Cary, NC, USA).

Results

Study Population

A total of 163 infants were enrolled and randomized into the trial (83 test formula, 80 control formula; Figure 4.3). Considering that the dropout rate was much lower than 30%, the minimum number of completed subjects needed to reach statistical power (n = 66 per treatment group) was achieved earlier than anticipated; therefore, the recruitment was ended before 95 infants were enrolled per treatment group. Of the 163 infants recruited, 142 infants completed the study (72 test formula, 70 control formula), while 21 infants (11 test formula, 10 control formula) discontinued the study. The reasons for discontinuation for each study group can be seen in Figure 4.3.

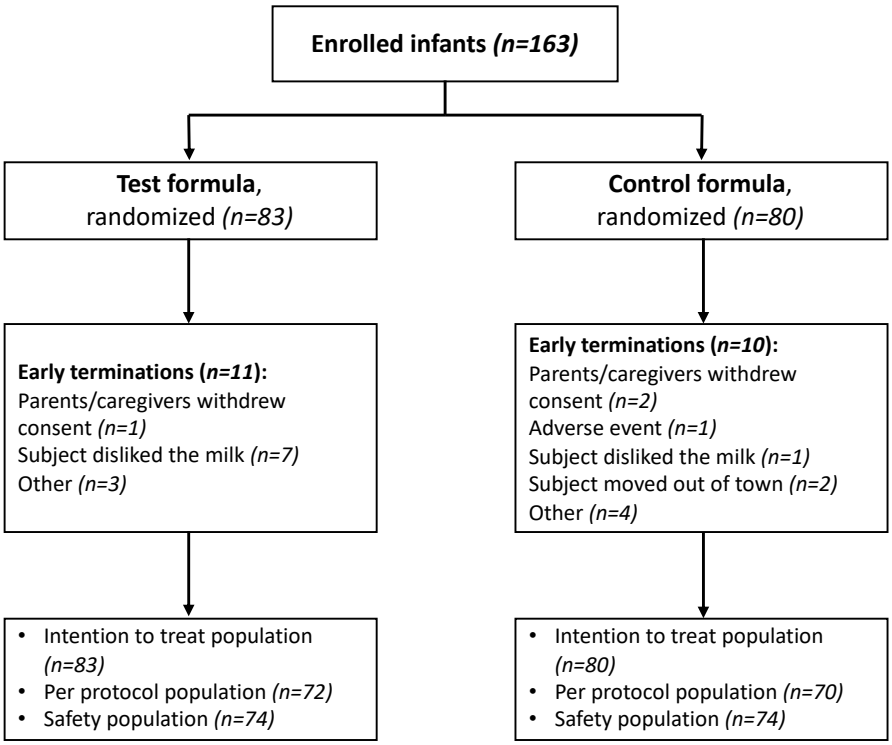

Figure 4-3. Study flowchart and subjects' disposition.

Test formula: Partially hydrolyzed whey infant formula; control formula: Standard intact protein formula.

Demographic, perinatal, and birth characteristics were comparable between the groups, except for years of maternal education (Table 4.6). Baseline characteristics also did not differ between the groups except for weight at baseline, indicating that infants in the control group had a higher weight at baseline than infants in the test group (Table 4.6).

Table 4-6. Demographic, perinatal, and baseline characteristics of study infants.

	Group	
	Test (*N* = 83)	**Control (*N* = 80)**
Infant characteristics		
Age at baseline (days), mean (SD)	66.9 (7.5)	67.1 (7.5)
Gender (female), *n* (%)	41 (49.4)	39 (48.8)
Weight at baseline (g), mean (SD)	5223 (694)[1]	5443 (639)
Length at baseline (cm), mean (SD)	59.12 (2.34)	59.26 (2.94)
Head Circumference at baseline (cm), mean (SD)	38.90 (1.31)	38.74 (1.23)
Birth weight (g), mean (SD)	3206 (398)	3159 (392)
Gestational age (weeks), mean (SD)	38.3 (1.1)	38.3 (1.1)
Caesarean delivery, *n* (%)	55 (66.3)	52 (65.0)
Maternal characteristics		
Age at baseline (years), mean (SD)	32.9 (6.4)	32.7 (5.8)
Parity (primiparous), *n* (%)	41 (49.4)	34 (42.5)
BMI at baseline (kg/m^2), mean (SD)	26.03 (4.74)	27.07 (5.07)
Education, *n* (%)		
≤12 years	28 (33.7) [1]	29 (36.2)
13–16 years	53 (63.9) [1]	40 (50.0)
>16 years	2 (2.4) [1]	11 (13.8)
Smoking during pregnancy, *n* (%)	22 (26.5)	16 (20.0)
Single pregnancy, *n* (%)	75 (90.4)	72 (90.0)

[1] p < 0.05. Test: Partially hydrolyzed whey infant formula; control: Intact protein formula; N: Number of subjects in analysis population; SD: Standard deviation; BMI: Body mass index.

Weight Gain and Growth

In the PP population, the adjusted mean (SE) weight gain during the three-month intervention period was 24.06 (2.64) g/day for infants fed the test formula and 24.54 (2.51) g/day for those fed the control (Table 4.7). The mean difference (95% CI) in weight gain between groups was -0.474 (-2.460, 1.512) g/day, with the lower limit of the 95% CI above the predefined non-inferiority margin of -3 g/day, rejecting the null hypothesis and indicating a similar weight gain in the two groups. Results were similar in the ITT population.

Table 4-7. Weight gain of study infants from baseline to the 3rd follow-up.

Population	Group	Weight Gain (g/d) Baseline—3rd Follow-Up	Difference between Groups (Test vs. Control)		*p*-Value
		LS mean (SE)	Estimate	95% CI	
PP	Test (*n* = 72)	24.06 (2.635)	−0.474	−2.460, 1.512	0.637
	Control (*n* = 70)	24.54 (2.513)			
ITT	Test (*n* = 83)	23.91 (2.789)	−0.641	−2.480, 1.399	0.535
	Control (*n* = 80)	24.55 (2.659)			

Test: Partially hydrolyzed whey infant formula; control: Intact protein formula; PP: Per protocol; ITT: Intention to treat; CI: Confidence interval; LS mean: Least squares mean; SE: Standard error.

Regarding the secondary outcomes, in the PP population, there were no significant differences between the two groups at any follow-up visit in weight, length, head circumference, and BMI (Supplementary file 4). Furthermore, no treatment effect over time was observed for any of those indices during the three-month intervention period (Supplementary file 4). Similar results were obtained in the ITT population (Supplementary file 5). Regarding gains in weight (in g/day) from baseline to the 1st or 2nd follow-up visits, no differences were observed between the two groups (Supplementary file 6). Likewise, no differences were found for gains in length (in cm/day) between the two groups over the three-month period (from baseline to each of the three monthly follow-up assessments; Supplementary file 6). Gains in head circumference (in cm/day) were slightly lower in the test group compared to the control from baseline to the 1st follow-up visit, but no differences were observed between the two groups thereafter (from baseline to the 2nd and 3rd follow-up assessments; Supplementary file 6). All the above findings were consistent between the PP and ITT populations.

Similarly, mean weight-for-age, length-for-age, head circumference-for-age, and BMI-for-age Z-scores did not di er between the two groups at any follow-up visit. Only weight-for-length Z-scores were slightly lower in the test group compared to the control at the 1st follow-up visit, but no differences were observed between the two groups thereafter. Results were again similar in the ITT population. Supplementary file 7 presents the relevant Z-scores of both groups during

the study period in comparison with the WHO growth standards for female and male infants based on the crude (unadjusted) data. All Z-scores were tracked closely with the WHO growth standards.

Formula Intake and Safety Parameters

Infants in the control group had a higher weekly formula consumption (~ +10.5%) compared to infants in the test group at all three follow-up measurements (Table 4.8). However, when the daily formula intake was corrected for body weight, no differences were observed between the two groups at all time points (Table 4.8).

Overall, 16 AEs occurred in the total study cohort, half of which (n = 8) occurred in the test formula group and half of which (n = 8) occurred in the control formula group. All the AEs and SAEs were unrelated to the intervention indicating no formula related risk (Supplementary file 8).

Discussion

The present study demonstrated a non-inferior weight gain between infants consuming a whey-based PHF and infants consuming a standard IPF during the three-month trial duration. Moreover, no differences were observed between the two groups on any growth measurements (weight, length, head circumference, and BMI), while overall growth trajectories were within the normal range based on WHO growth standards (WHO, 2009). The two formulas used in the current study were similar with regards to macro-nutrients, apart from the protein fraction, and were therefore isocaloric, providing 66 kcal per 100 mL. The slight differences in galacto-oligosaccharides, which are non-digestible oligosaccharides, and some micro-nutrients could not have affected the weight gain of infants. Therefore, as hypothesized, the absence of differences on growth outcomes between the two formula groups suggests that substituting intact protein with partially hydrolyzed protein in IF is safe and supports appropriate growth in healthy infants.

Table 4-8. Formula intake at each follow-up visit by study group.

Daily Formula Intake by Body Weight (mL/g/d)						
	PP Population			**ITT Population**		
Study Visit	**Test**	**Control**		**Test**	**Control**	
	LS Mean (95% CI)	**LS Mean (95% CI)**	***p*-Value**	**LS Mean (95% CI)**	**LS Mean (95% CI)**	***p*-Value**
Follow-up 1	1.00 (0.96, 1.04)	1.02 (0.97, 1.06)	0.651	1.00 (0.96, 1.05)	1.01 (0.97, 1.05)	0.807
Follow-up 2	0.95 (0.90, 0.99)	0.98 (0.94, 1.02)	0.268	0.95 (0.90, 0.99)	0.98 (0.94, 1.03)	0.239
Follow-up 3	0.92 (0.89, 0.96)	0.93 (0.89, 0.97)	0.808	0.92 (0.89, 0.96)	0.93 (0.89, 0.97)	0.808
Weekly formula intake (mL)						
	PP population			**ITT population**		
	Test	**Control**		**Test**	**Control**	
	Median	**Median**	***p*-value**	**Median**	**Median**	***p*-value**
Follow-up 1	5757.5	6492.5	<0.001	5797.5	6455.0	0.001
Follow-up 2	6107.5	6880.0	<0.001	6107.5	6860.0	<.001
Follow-up 3	6420.0	7040.0	0.002	6420.0	7040.0	0.002

Test: Partially hydrolyzed whey infant formula; control: Intact protein formula; PP: Per protocol; ITT: Intention to treat; SE: Standard error.

Regarding the primary outcome, the current results are consistent with previous studies. In the study by Wu et al. (Wu et al., 2017), no differences were observed in daily weight gain in healthy term infants fed a PHF compared to infants fed an IPF or breast milk from enrolment to the 7th and 13th week of age. Florendo et al. (Florendo et al., 2009) compared the effects of a standard non-hydrolyzed whey–casein formula to a preterm PHF for three weeks. No differences in daily weight gain were observed between the two groups during the 3-week study duration. In the German Infant Nutritional Intervention Study (GINI) (Rzehak et al., 2009), four different types of formulas were assessed, as well as a breast milk reference group; these formulas were either a whey PHF, an extensively hydrolyzed whey formula, an extensively hydrolyzed casein formula, or a regular IPF. Weight gain during the first four and six months of life showed no differences in infants with atopic heredity who consumed either breast milk or one of the formula groups, except for the extensively hydrolyzed casein formula which showed a transient lower weight gain. Despite the diverse study designs and IF used, it has been shown overall that no differences in weight gain were observed when healthy infants were fed either PHF or regular IPF during early infancy.

The findings of the current study on secondary outcomes, i.e., weight, length, head circumference, and BMI showed no differences between the test and control groups at all three time points. These findings are also in line with the results reported for those indices by Wu et al. (Wu et al., 2017), Florendo et al. (Florendo et al., 2009), and the GINI study (Rzehak et al., 2009) described above. Similar findings were also reported in other studies (Exl et al., 2000; Sun et al., 2015). Although diffcult to directly compare due to methodological variations, previous studies and current results collectively suggest that weight, length, head circumference, and BMI of infants fed either protein hydrolysate-based formulas or regular IPF do not show any differences during the first months of life.

Regarding mean Z-scores (weight-for-age, length-for-age, head circumference-for-age, weight-for-length, and BMI-for-age), the current study found no differences between the two study groups during the three-month period. Furthermore, all mean Z-scores were within the normal range based on WHO growth standards (WHO, 2009). Again, consistent results have been reported by previous studies as mentioned above (Exl et al., 2000; Florendo et al., 2009; Rzehak et al., 2009; Sun et al., 2015; Wu et al., 2017). However, in the study by Mennella et al. (Mennella et al., 2011), Z-scores trajectories across infants aged 2.5 to 7.5 months showed significantly higher weight-for-age Z-scores in the infants fed a regular IPF compared to infants fed a PHF.

Weight gain was accelerated in the former, whereas it was normative in the latter. Still, the differences observed in weight gain rates in this study could be attributed to the difference in the amount of formula consumed between the two study groups, since infants in the protein hydrolysate group consumed less formula to satiation than did regular formula-fed infants across the study period (Mennella et al., 2011).

Regarding formula intake, a significant group effect was observed in the present study, with infants in the test group consuming less formula than infants in the control group at each monthly follow-up assessment. This phenomenon, also observed in the study by Menella et al. (Mennella et al., 2011), could be attributed to the sensory characteristics of the two formulas, as infants may dislike the taste of protein hydrolysates, occurring due to the increased levels of free amino acids and small peptides with a bitter taste, and consequently consume less. This is further supported by the fact that the main reason for dropping out of the study in the test group was that infants disliked the test formula. Still, the overall drop-out rate was much lower than anticipated. Furthermore, it has been shown that the sooner a hydrolysate-based formula is introduced in an infant's diet, the more accepted it is by the infant (Mennella et al., 2004). Therefore, considering that infants in the present study had a mean age of 67 days at baseline, the test formula might have not been equally accepted by the infants as the control formula. Another potential explanation could be that hydrolyzed proteins have been shown to promote satiation signals and stimulate earlier meal termination in infants who consume protein hydrolysate-based formulas (Diepvens et al., 2008; Foltz et al., 2008). Nevertheless, the lower formula intake observed in infants consuming the test formula did not affect weight gain or other growth outcomes at any time point compared to the control formula in the current study, and supported normative growth based on WHO growth standards (WHO, 2009).

Among the strengths of the current study are the double-blind study design and the standardized procedure followed for data collection. Specifically, recruitment was performed by several pediatricians, but infants' growth was prospectively assessed by the same pediatrician who enrolled them in the study, during the entire study period. Still, the large number of pediatricians involved in the study could introduce some variation in the measurements performed. To ensure comparability of the anthropometric data obtained among sites, all pediatricians were trained to follow the same standardized procedures for anthropometrics, while intra- and inter-observer reliability was also periodically assessed. Another strength of the present study was that, as

described in the methods section, different randomization tables were created for each pediatrician to ensure that infants would be appropriately randomized across treatments within each site.

Conclusions

The current study demonstrated that weight gain, as well as other growth outcomes did not differ between infants consuming the whey-based PHF and those consuming the IPF. All the Z-score indices obtained were within the normal range of WHO growth standards. Based on these results, it can be concluded that the IF with partially hydrolyzed protein supports appropriate growth in healthy term infants.

Supplementary file 1. Inclusion and exclusion criteria

Inclusion criteria:

- Full-term, healthy infants (born at gestational age ≥37 weeks) in the general population
- Appropriate for gestational age birthweight (i.e. 10th centile ≤ Birth weight ≤ 90th centile)
- Boys and girls
- Age at enrolment (baseline measurement): between 55 and 80 days of age
- Exclusively formula fed two weeks before inclusion
- Exclusively formula fed during the entire intervention period
- Parents agreeing to initiate complementary feeding after finalization of the study (endpoint measurements at ~5.5 months of age)
- Being available for follow up until the age of approximately 5.5 months
- Written informed consent

Exclusion criteria:

- Severe acquired or congenital diseases, mental or physical disorders including cow's milk protein allergy (CMPA), lactose intolerance and diagnosed medical conditions that are known to affect growth [i.e. gastrointestinal (GI) disorders]
- Illness at screening/inclusion
- Incapability of parents to comply with the study protocol
- Participation in another clinical trial
- Unwillingness to accept the formula supplied by the study as the only formula for their child during study participation

Supplementary file 2.

Table S. 4-2. Analytical composition of the study formulas (per 100 ml).

	Test formula	**Control formula**
Energy (kcal)	66	66
Intact protein (g)		1.4
Casein		0.57
Whey		0.85
Whey protein hydrolysate (g)	1.6	
Fat (g)	3.5	3.5
DHA (mg)	6.9	6.9
Arachidonic Acid (mg)	6.9	6.9
Carbohydrates	7.0	7.0
GOS (g)	0.2	0.4
Calcium (mg)	50	56
Phosphorus (mg)	30	31
Sodium (mg)	20	23
Iron (mg)	0.78	0.77
Copper (μg)	50	47
Potassium (mg)	65	79
Magnesium (mg)	6	6.4
Manganese (μg)	17	16
Zinc (mg)	0.60	0.60
Chlorine (mg)	42	47
Iodine (μg)	10	9
Selenium (μg)	1.7	2.5
Vitamin A (μg-RE)	70	74
Vitamin D (μg)	1.2	1.1
Vitamin E (mg)	1.3	1.7
Vitamin K (μg)	5.1	6.2
Vitamin B1 (μg)	59	57
Vitamin B2 (μg)	91	78

Niacin mg	0.47	0.49
Vitamin B6 (μg)	39	58
Vitamin B12 (μg)	0.16	0.16
Folic acid (μg)	10	11
Pantothenic acid (μg)	0.33	0.40
Biotin (μg)	1.4	1.7
Vitamin C (mg)	9.1	11
Nucleotides (mg)	3.25	3.25
Taurine (mg)	6	7.3
Choline (mg)	14	21
Inositol (mg)	3.9	4.4
Carnitine (mg)	1.7	1.6

Test formula: partially hydrolyzed whey infant formula; control formula: intact protein formula; DHA: Docosahexaenoic acid; GOS: galacto-oligosaccharides.

Supplementary file 3. Primary and secondary outcome measures and statistical analysis.

The primary outcome was weight gain (g/day) calculated as the difference in infant weight between the baseline and the 3rd follow-up visit, divided by the number of days between these visits. Secondary outcomes included other anthropometric indices assessed at each follow-up visit: weight (g), length (cm), head circumference (cm), BMI (kg/m2) and their Z-scores [based on the World Health Organisation (WHO) child growth standards][1]. Furthermore, recumbent length gain (cm/day) and head circumference gain (cm/day) from baseline to each one of the follow-up visits were calculated and compared between the two groups, while weight gain (g/day) between the baseline and the 1st and 2nd follow-up visits was also evaluated. Infants' anthropometrics were measured in triplicates at baseline and at every monthly follow-up visit by the paediatricians, following standardized procedures. Specifically, the weight of each infant was recorded by weighing it three times wearing nothing but a clean diaper, on calibrated electronic scales, and if the pair-wise difference of the three measurements was more than 100 g, an additional measurement was performed. Recumbent length was measured three times using a standard measuring board, and in case of a deviation of more than 0.7 cm an additional measurement was performed. Non-stretchable slotted insertion tape was used to measure head circumference and if the pair-wise difference of the three measurements was more than 0.5 cm, an additional measurement was performed.

For the demographic, perinatal and baseline characteristics of study participants, P-values for the between groups difference (test minus control) for age at baseline were calculated using Wilcoxon Rank Sum Test; P-values for the between groups difference (test minus control) for all anthropometric indices at baseline were calculated using two Independent Sample t Tests; P-values for the between groups difference (test minus control) for all other characteristics were calculated using Cochran-Mantel-Haenszel test. The primary outcome was analyzed using an Analysis of Covariance model with study formula as fixed factor, adjusting for baseline weight, sex, antibiotics use, illnesses independent of the formula (related/non-related adverse events), smoking in the home environment, birth weight, maternal pre-pregnancy BMI,

father's current BMI, existence of gestational diabetes and if it was untreated, formula intake. The secondary outcomes were analyzed using Mixed Models Repeated Measures analysis with study formula and visit as fixed factors and adjusting for baseline anthropometry, sex, smoking in the home environment, antibiotics use, illnesses independent of the formula, birth weight, maternal pre-pregnancy BMI, father's current BMI, existence of gestational diabetes and whether it was untreated, formula intake and significant interactions of these covariates with visit and/or study formula, with Kenward-Roger degrees of freedom and autoregressive covariance structure. For comparison of weekly formula consumption between the two groups, a Wilcoxon Rank Sum Test was used, while for analysis of the daily formula intake by body weight (mL/g/d) between the two groups an Analysis of Variance model with study formula as fixed factor was used.

[1]*WHO Multicentre Growth Reference Study Group. WHO Child Growth Standards : Length/Height-for-Age, Weight-for-Age, Weight-for-Length, Weight-Forheight and Body Mass Index-for-Age : Methods and Development.; 2006. doi:10.4067/S0370-41062009000400012*

Supplementary file 4.

Table S. 4-3. Weight, length, head circumference and BMI at each follow-up visit by study group in the PP population.

Study Visit	Test (n=72)	Control (n=70)	Difference of means[3]			
	LS Mean (SE)	LS Mean (SE)	Difference	95% CI	P-value[1]	P-value[2]
Weight, g						
Follow-up 1	6224 (158)	6221 (149)	2.74	-138.23, 143.71	0.969	
Follow-up 2	6902 (158)	6997 (149)	-95.02	-236.04, 46.01	0.185	0.330
Follow-up 3	7456 (158)	7560 (149)	-104.05	-244.99, 36.89	0.147	
Length, cm						
Follow-up 1	64.10 (0.57)	63.64 (0.54)	0.46	-0.07, 0.99	0.090	
Follow-up 2	66.69 (0.57)	66.51 (0.55)	0.18	-0.35, 0.71	0.499	0.283
Follow-up 3	69.21 (0.57)	69.08 (0.55)	0.13	-0.40, 0.66	0.628	
BMI, kg/m^2						
Follow-up 1	40.15 (0.27)	40.27 (0.25)	-0.13	-0.37, 0.12	0.307	
Follow-up 2	41.31 (0.27)	41.46 (0.25)	-0.15	-0.39, 0.10	0.238	0.244
Follow-up 3	42.27 (0.27)	42.39 (0.25)	-0.12	-0.36, 0.13	0.349	
Head circumference, cm						
Follow-up 1	15.05 (0.43)	15.43 (0.41)	-0.38	-0.77, 0.01	0.055	
Follow-up 2	15.48 (0.43)	15.85 (0.41)	-0.37	-0.76, 0.02	0.065	0.064
Follow-up 3	15.55 (0.43)	15.82 (0.41)	-0.27	-0.66, 0.12	0.171	

[1] *between groups difference per time point*
[2] *average treatment effect over time*
[3] *difference in LS means between test and control formula*
Test: partially hydrolyzed whey infant formula; control: intact protein formula; PP: per protocol; CI: confidence interval; LS mean: least squares mean; SE: standard error; BMI: body mass index.

Supplementary file 5.

Table S. 4-4. Weight, length, head circumference and BMI at each follow-up visit by study group in the ITT population.

Study Visit	Test (n=83)	Control (n=80)	Difference of means[3]			
	LS Mean (SE)	LS Mean (SE)	Difference	95% CI	P-value[1]	P-value[2]
Weight, g						
Follow-up 1	6217 (157)	6217 (148)	0.44	-136.41, 137.28	0.995	
Follow-up 2	6891 (157)	6989 (148)	-98.76	-236.38, 38.87	0.158	0.278
Follow-up 3	7450 (157)	7564 (148)	-114.25	-252.55, 24.04	0.105	
Length, cm						
Follow-up 1	63.99 (0.57)	63.60 (0.54)	0.39	-0.13, 0.91	0.138	
Follow-up 2	66.57 (0.57)	66.47 (0.54)	0.10	-0.42, 0.62	0.703	0.459
Follow-up 3	69.10 (0.57)	69.08 (0.54)	0.03	-0.50, 0.55	0.918	
BMI, kg/m^2						
Follow-up 1	15.09 (0.43)	15.43 (0.41)	-0.34	-0.72, 0.03	0.075	
Follow-up 2	15.51 (0.43)	15.84 (0.41)	-0.34	-0.72, 0.04	0.083	0.086
Follow-up 3	15.58 (0.43)	15.82 (0.41)	-0.24	-0.62, 0.14	0.214	
Head circumference, cm						
Follow-up 1	40.10 (0.27)	40.26 (0.25)	-0.16	-0.40, 0.08	0.187	
Follow-up 2	41.26 (0.27)	41.43 (0.25)	-0.17	-0.41, 0.07	0.153	0.144
Follow-up 3	42.23 (0.27)	42.37 (0.25)	-0.14	-0.38, 0.10	0.239	

[1] between groups difference per time point
[2] average treatment effect over time
[3] difference in LS means between test and control formula
Test: partially hydrolysed whey infant formula; control: intact protein formula; ITT: intention to treat; CI: confidence interval; LS mean: least squares mean; SE: standard error; BMI: body mass index.

Supplementary file 6.

Table S. 4-5. Gains in weight, length and head circumference at each follow-up visit by study group.

	Population	Group	LS mean (SE)	Difference between groups (Test vs. Control)		P-value
				Estimate	95% CI	
Weight gain (g/d) Baseline – 1st follow-up	PP	Test	26.28 (2.78)	-1.71	-4.24, 0.82	0.184
		Control	27.99 (2.63)			
	ITT	Test	26.17 (2.84)	-1.60	-4.11, 0.90	0.207
		Control	27.78 (2.68)			
Weight gain (g/d) Baseline – 2nd follow-up	PP	Test	24.60 (2.78)	-1.56	-4.10, 0.97	0.225
		Control	26.16 (2.63)			
	ITT	Test	24.38 (2.84)	-1.63	-4.15, 0.89	0.203
		Control	26.01 (2.68)			
Length gain (cm/d) Baseline – 1st follow-up	PP	Test	0.137 (0.012)	0.01	-0.00, 0.02	0.052
		Control	0.127 (0.011)			
	ITT	Test	0.135 (0.012)	0.01	-0.00, 0.02	0.104
		Control	0.126 (0.011)			
Length gain (cm/d) Baseline – 2nd follow-up	PP	Test	0.127 (0.012)	0.00	-0.01, 0.01	0.518
		Control	0.124 (0.011)			
	ITT	Test	0.125 (0.012)	0.00	-0.01, 0.01	0.820
		Control	0.124 (0.011)			
Length gain (cm/d) Baseline – 3rd follow-up	PP	Test	0.124 (0.012)	0.00	-0.01, 0.01	0.494
		Control	0.120 (0.011)			
	ITT	Test	0.122 (0.012)	0.00	-0.01, 0.01	0.694
		Control	0.120 (0.011)			
HC gain (cm/d) Baseline – 1st follow-up	PP	Test	0.041 (0.006)	-0.01	-0.01, -0.00	**0.040**
		Control	0.047 (0.005)			
	ITT	Test	0.040 (0.006)	-0.01	-0.01, -0.00	0.017
		Control	0.046 (0.005)			
HC gain (cm/d)	PP	Test	0.038 (0.006)	-0.00	-0.01, 0.00	0.375

Baseline – 2nd follow-up	ITT	Control	0.040 (0.005)			
		Test	0.037 (0.006)	-0.00	-0.01, 0.00	0.241
	PP	Control	0.040 (0.005)			
		Test	0.035 (0.006)	-0.00	-0.01, 0.00	0.843
HC gain (cm/d)		Control	0.035 (0.005)			
Baseline – 3rd follow-up	ITT	Test	0.034 (0.006)	-0.00	-0.01, 0.00	0.681
		Control	0.035 (0.005)			

Figures in bold indicate statistically significant P-values.
Test: partially hydrolysed whey infant formula; control: intact protein formula; PP: per protocol; ITT: intention to treat; CI: confidence interval; LS mean: least squares mean; SE: standard error.

Supplementary file 7.

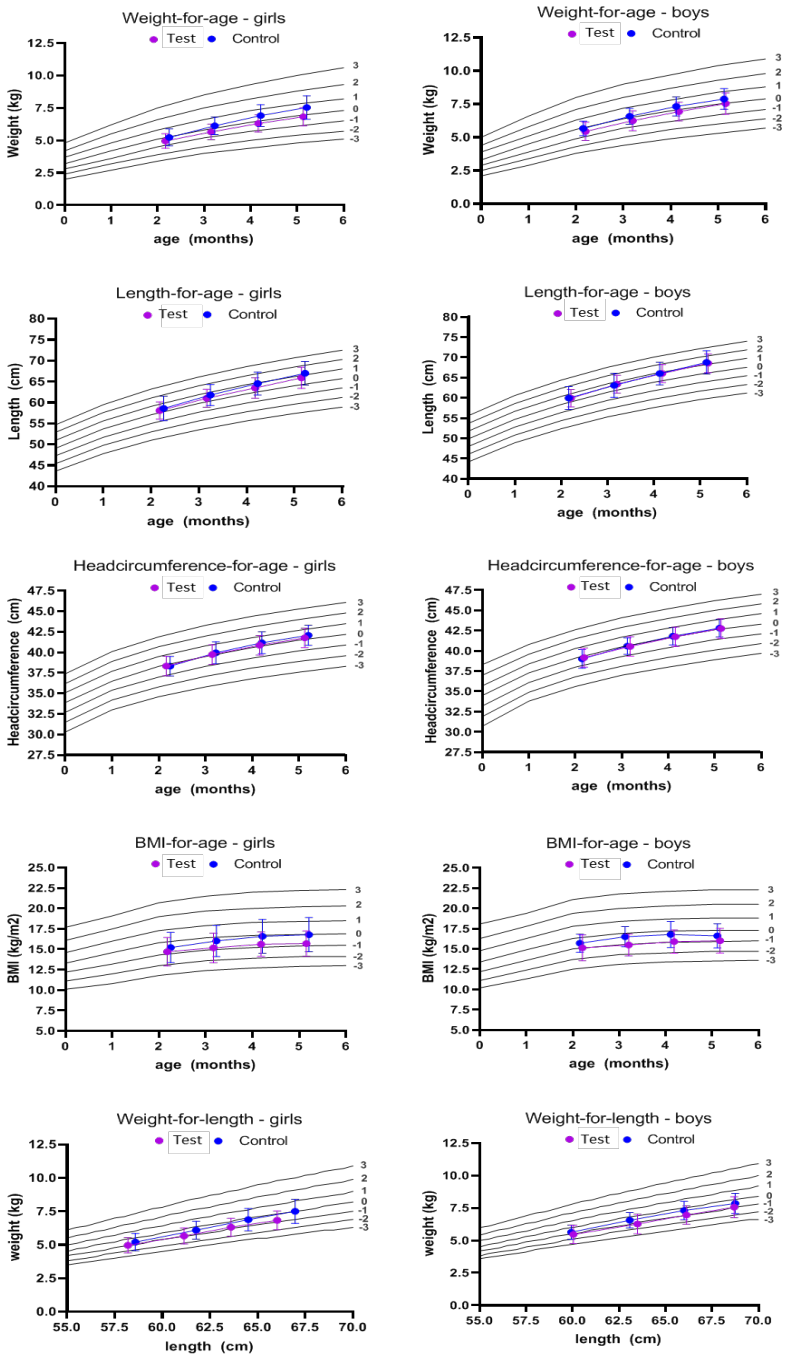

Figure S. 4-1. Anthropometric measurements expressed as Z-scores for weight-for-age, length-for-age, head circumference-for-age, weight-for-length and BMI-for-age during the study period in comparison with the World Health Organization growth standards for female and male infants.

Test: partially hydrolyzed whey infant formula; control: standard intact protein formula.

Supplementary file 8.

Table S. 4-6. Overview of adverse events and serious adverse events that occurred during the trial.

		Safety population*		
		Group		
Category	**Statistic**	**Test (N=74)**	**Control (N=74)**	**Total (N=148)**
Adverse Events	k	8	8	16
	n (%)	7 (9.5)	7 (9.5)	14 (9.5)
Related Adverse Events	k	0	0	0
	n (%)	0 (0.0)	0 (0.0)	0 (0.0)
Discontinued Adverse Events	k	0	0	0
	n (%)	0 (0.0)	0 (0.0)	0 (0.0)
Serious Adverse Events	k	5	2	7
	n (%)	4 (5.4)	1 (1.4)	5 (3.4)
Related Serious Adverse Events	k	0	0	0
	n (%)	0 (0.0)	0 (0.0)	0 (0.0)
Discontinued Serious Adverse Events	k	0	0	0
	n (%)	0 (0.0)	0 (0.0)	0 (0.0)

Test: partially hydrolysed whey infant formula; control: intact protein formula; N: number of subjects in analysis population; n: number of subjects with at least one event; %: percentage of subjects with at least one event, k: number of events.

**Safety population: all infants of the ITT data analysis set minus the ones who did not consume any formula at all.*

MANUSCRIPT 3: Comfort related parameters in healthy infants fed with partially hydrolysed formula or intact protein formula.

Introduction

Appropriate infant nutrition is the cornerstone of a child's healthy growth and development as the nutrition in early life sets the infant on a developmental trajectory for the rest of his or her life (Fragkou et al., 2021; Haschke et al., 2019). Human milk is recognized as complete and the best source of nutrients for infants. However, when breastfeeding is not available, infant formulas designed to provide all necessary nutrients, are the only suitable alternative.

In an attempt to optimize infant nutrition, manufacturers have been modifying formulas' content and manufacturing processes to better suit infant needs (Green Corkins and Shurley, 2016). An overall trend over the years has been to approximate the content and functionality of human breast milk. However, there are variations in the composition and manufacturing processes of different infant formulas (Green Corkins and Shurley, 2016).

The protein fraction in infant formulas can be either intact, partially or extensively hydrolysed or amino acid based (Drapala et al., 2016). Partially hydrolysed formulas (pHF) were initially recommended for infants at risk of developing cow's milk protein allergy, as the hydrolysis decreases the antigenicity of proteins (Hernández-Ledesma et al., 2014). More recently, formulas containing partial hydrolysates are also promoted for healthy infants in addition to those with Functional Gastrointestinal Disorders (FGIDs), under the assumption that accelerated digestion rates will benefit overall digestive comfort (Vandenplas et al., 2019). Several studies have been conducted in the past to justify this claim, however test formulas usually are significantly different from the control formulation in more than just the protein type, making it challenging to draw conclusions specifically for the impact of pHF on infant digestive comfort (Huang et al., 2021; Picaud et al., 2020; Savino et al., 2005; Slavin, 2013).

Making matters more complicated, aside from the individual ingredients in the formulas, manufacturing processes may also have a significant impact on a formula's digestibility and resulting gastrointestinal (GI) comfort (Sheng et al., 2020). The heating stages of the infant formula manufacturing process are critical to ensuring the quality and safety of the final product. However, this heat-intensive processing can change the structure of milk proteins in several ways, resulting in the denaturation and aggregation of the protein and chemical modifications of

its amino acids (van Lieshout et al., 2020). High content of lactose and lysine make the proteins in infant formula more susceptible to glycation. The resulting blocked lysine decreases the bioavailability of amino acids in the formula and results in poor intestinal absorption (van Lieshout et al., 2020). This may potentially affect the growth and development of infants and moreover lead to potential microbiome changes and gas formation that may further result in digestive discomfort (Diether and Willing, 2019; van Lieshout et al., 2020).

The primary objective of the current study as reported earlier, was to evaluate the weight gain of healthy term infants consuming a partially hydrolysed whey-based infant formula compared to a commercially available minimally processed infant formula (control) with intact protein over a period of 3 months (Karaglani et al., 2020). In this paper, we report the analysis of tertiary outcomes covering digestive comfort parameters from the same study of both infant formulas, in combination with a post-hoc analysis of corresponding growth data. Both products were closely matched in composition except for the protein fraction and level of galacto-oligosaccharides (GOS).

Methods

Study design and population

This study was a double-blind, randomized controlled trial with two treatment arms: the test group consuming the pHF and the control group consuming the IPF. Both formulas are commercially available. The study was conducted in 163 healthy, full-term, exclusively formula-fed infants, between 55 and 80 days of age at baseline, who received one of the two formulas at random (Karaglani et al., 2020).

The study protocol, information letter to parents/legal guardians and written informed consent forms were approved by Harokopio University's Ethics Committee (approval code: 62/03-07-2018). The study was conducted in accordance with the guidelines of the Declaration of Helsinki and the International Conference on Harmonization (ICH) guidelines on Good Clinical Practice (GCP) and was registered in the Netherlands Trial Registry [identifier: NL7378 (NTR7586)].

Study procedures and intervention

Upon inclusion in the study, subjects were randomized to one of four coded products representing the two study formulas. Randomization was performed centrally, at Harokopio

University, by a designated and trained research assistant based on computer-generated schemes.

Formulas were provided for free to the participating families during the three-month study period and were used as the sole source of nutrition for the participating infants. Formula consumption was ad libitum but a feeding table in the "Parent Information Brochure" supported a correct consumption of the study products.

The nutritional compositions of the infant formulas used in this study were similar with regards to macro-nutrients, apart from the protein fraction (Supplementary file 1). Both infant formulas were bovine milk-based and were produced in the Netherlands by Friesland Campina and packed in blank tins of 400g each with a specific identification code at the bottom. All powder properties were identical between the test and control formulas. Parents/legal guardians, investigators and study support staff were blinded to the formulas. Data analyses were performed with the study groups coded and the code was not broken until the database was locked.

Tertiary outcome measures and post-hoc analysis

Data were collected at four visits over a period of 3 months. Gastrointestinal comfort was assessed from data collected via two questionnaires. The Infant Gastrointestinal Symptoms Questionnaire (IGSQ) was completed at each study visit (Riley et al., 2015). The questionnaire is subdivided into five different domains: stooling, spitting up/vomiting, crying, fussiness, and gassiness. It is a 13-item index of parent-reported infant digestion and elimination behaviours, covering parameters including stooling (two questions), spitting up/vomiting (four questions), crying and fussiness (five questions), and gassiness (two questions). Each item is scored on a scale of one to five with higher values indicating greater GI distress. A composite IGSQ score was derived from summing the individual scores with a possible range of 13 to 65, where higher values indicate greater GI distress and values ≤23 indicate no digestive distress. A similar principle was applied to subsections of the IGSQ covering different dimensions of GI discomfort.

The second questionnaire administered was the Amsterdam Infant Stool Scale (AISS) (Bekkali et al., 2009). This was completed by parents any time the infant defecated in the three days prior to all follow-up visits. The questionnaire recorded stool frequency, volume, consistency and colour. On the instance the AISS was not filled out for a certain day, it was assumed that the infant did not have any bowel movements on that particular day.

Additionally, growth was compared against World Health Organization (WHO) standards (WHO, 2009). Anthropometric indices assessed at each follow-up visit included weight (g), length (cm), body mass index (BMI) (kg/m^2) and their Z-scores.

Sample size and statistical analysis

Details on sample size determination are based on the non-inferiority weight gain between infants receiving the two formulas as previously published in Karaglani et al. (Karaglani et al., 2020). The GI comfort parameters were analysed using independent t-tests and mixed model repeated measures analysis [IBM SPSS Statistics for Windows, version 24 (IBM Corp., Armonk, N.Y., USA)] with visit and study formula as fixed factors. Analysis of the AISS and IGSQ were adjusted for potential covariates (sex, antibiotic use, illness unrelated to the study formula and smoking in the home environment). Estimates for the IGSQ scores were additionally adjusted for baseline IGSQ. Associations of GI parameters with parameters of growth and milk intake were tested using Pearson's correlation coefficient (r). All analyses were performed for the per-protocol (PP) population only.

Results

Study population

A total of 163 infants were enrolled and randomized into the trial. Of these, 142 infants completed the study (72 pHF group and 70 IPF group). The demographic, perinatal and baseline characteristics of the infants in the two groups and their parents are reported in Table 4.9.

Table 4-9. Demographic, perinatal and baseline characteristics of infants and parental characteristics in the pHF and IPF groups.

Infant characteristics	**pHF (N=72)**	**IPF (N=70)**
Age at baseline (days), mean (SD)	66.93 (7.828)	66.77 (7.435)
Gestational age (weeks), mean (SD)	38.24 (1.041)	38.21 (1.048)
Weight at birth (g), mean (SD)	3186.53 (382.870)	3156.57 (406.536)
Length at birth (cm), mean (SD)	50.13 (1.936)	50.44 (2.188)
Weight at baseline (g), mean (SD)	5179.69 (699.015)	5482.036 (617.566)
Length at baseline (cm), mean (SD)	59.10 (2.365)	59.39 (2.918)

Head circumference at birth (cm), mean (SD)	34.22 (1.206)	34.20 (1.213)
BMI at birth (kg/m^2), mean (SD)	12.67 (1.242)	12.38 (1.148)
Start full formula feeding (days), mean (SD)	20.31 (17.271)	26.51 (17.425)
IGSQ scores at baseline, mean (SD)		
Total score	26.46 (6.807)	27.59 (5.835)
Stooling	3.18 (1.577)	3.76 (1.789)
Spitting up/vomiting	7.84 (3.224)	8.41 (3.369)
Crying	5.44 (2.500)	5.07 (2.267)
Fussiness	3.49 (1.950)	3.54 (1.800)
Gassiness	6.50 (1.712)	6.80 (1.774)
Maternal characteristics		
Age at baseline (years), mean (SD)	32.82 (6.447)	32.47 (5.434)
Weight before pregnancy (kg), mean (SD)	66.76 (14.406)	70.89 (18.175)
Height (cm), mean (SD)	163.97 (6.441)	165.56 (6.095)
BMI before pregnancy (kg/m^2), mean (SD)	24.79 (5. 018)	25.72 (5.779)
Weight at baseline (kg), mean (SD)	70.46 (14.323)	74.56 (16.046)
Paternal characteristics		
Age at baseline (years), mean (SD)	35.91 (7.308)	36.72 (5.805)
Weight at baseline (kg), mean (SD)	86.20 (14.726)	89.03 (17.734)
Height (cm), mean (SD)	178.38 (5.770)	178.23 (7.635)
BMI at baseline (kg/m^2), mean (SD)	27.13 (3.847)	27.91 (5.012)

pHF: Partially hydrolyzed whey infant formula; IPF: Intact protein formula; N: Number of subjects in analysis population; SD: Standard deviation; IGSQ: Infant Gastrointestinal Symptoms Questionnaire; BMI: Body mass index

GI comfort

The IGSQ scores of both groups are reported in Table 4.10. No significant differences were observed in the overall IGSQ score between the two groups. Both groups showed good digestive comfort outcomes, with no differences observed in any of the IGSQ items related to reflux, crying or fussiness between groups. Infants on the pHF were however reported to be significantly more gassy compared with those on IPF (p=0.002). In addition, infants in the pHF group reported lower consistency of stools than those in the IPF group (p=0.012).

Stooling was assessed in more depth using the AISS. Results from the AISS showed no differences in defecation frequency between the two groups. Table 4.11 reports the stooling patterns of the two groups, respectively. Consumption of the pHF resulted in larger and significantly looser ($p \leq 0.001$) stools. A difference in stool colour was also observed between the two groups ($p < 0.001$), with IPF associated with yellow colour while pHF resulted in green-coloured stools.

Table 4-10. IGSQ scores (overall and per domain) for the PP population at each follow-up visit according to study group.

Variables		Visit 2		Visit 3		Visit 4		p-value[1]
		pHF	IPF	pHF	IPF	pHF	IPF	
IGSQ (total and subdomains)		**N=72**	**N=70**	**N=72**	**N=70**	**N=72**	**N=70**	
Total IGSQ score (max 65)	mean (SE)	24.34 (0.69)	24.75 (0.69)	23.92 (0.70)	22.89 (0.69)	23.56 (0.70)	22.39 (0.71)	0.295
Stooling (max 10)	mean (SE)	2.57 (0.13)	2.89 (0.13)	2.35 (0.13)	2.67 (0.13)	2.36 (0.13)	2.54 (0.13)	**0.012**
Spitting up/vomiting (max 20)	mean (SE)	7.45 (0.36)	7.57 (0.37)	7.39 (0.36)	7.01 (0.37)	7.76 (0.36)	6.64 (0.37)	0.123
Crying (max 15)	mean (SE)	4.25 (0.20)	4.64 (0.21)	4.63 (0.20)	4.53 (0.21)	4.43 (0.20)	4.39 (0.21)	0.634
Fussiness (max 10)	mean (SE)	3.46 (0.20)	3.33 (0.21)	3.46 (0.20)	3.50 (0.21)	3.18 (0.20)	3.46 (0.21)	0.708
Gassiness (max 10)	mean (SE)	6.43 (0.19)	6.04 (0.20)	6.10 (0.19)	5.44 (0.20)	5.82 (0.19)	5.34 (0.20)	**0.002**

pHF: Partially hydrolyzed whey infant formula; IPF: Intact protein formula; N: Number of subjects in analysis population; IGSQ: Infant Gastrointestinal Symptoms Questionnaire; SE: Standard error.

[1] Mixed Model Repeated Measures (MMRM) analysis with study formula and visit as fixed factors, adjusted for baseline IGSQ scores, sex, smoking in the home environment, antibiotics use, and illnesses independent of the formula.

Table 4-11. Mean AISS scores (3-day average) for the PP population at each follow-up visit according to study group.

Variables		Visit 2		Visit 3		Visit 4		p-value[1]
		pHF	IPF	pHF	IPF	pHF	IPF	
AISS (3-day average)		**N=72**	**N=70**	**N=72**	**N=70**	**N=72**	**N=70**	
Daily stool frequency	mean (SE)	1.42 (0.08)	1.50 (0.08)	1.50 (0.08)	1.50 (0.08)	1.36 (0.08)	1.51 (0.08)	0.252
Daily stool volume	mean (SE)	3.32 (0.08)	3.22 (0.08)	3.36 (0.08)	3.15 (0.08)	3.37 (0.08)	3.05 (0.08)	**0.001**
Daily stool consistency	mean (SE)	1.56 (0.05)	2.03 (0.05)	1.60 (0.05)	1.99 (0.05)	1.99 (0.05)	2.01 (0.05)	**<0.001**
Daily stool colour	mean (SE)	2.77 (0.11)	1.66 (0.11)	2.94 (0.11)	1.76 (0.11)	2.89 (0.11)	1.65 (0.12)	**<0.001**

pHF: Partially hydrolyzed whey infant formula; IPF: Intact protein formula; N: Number of subjects in analysis population; AISS: Amsterdam Infant Stool Scale; SE: Standard error.

[1] *Mixed Model Repeated Measures (MMRM) analysis with study formula and visit as fixed factors, adjusted for sex, smoking in the home environment, antibiotics use, and illnesses independent of the formula.*

Weight gain and growth

Results of weight gain and growth of the infants throughout the study period were reported in Karaglani et al. (Karaglani et al., 2020). The weight gain recorded in the two groups was similar in the PP population. Similarly, there were no differences between the two groups for the mean weight-for-age, length-for-age and BMI-for-age Z-scores (Figure 4.4 and Supplementary files 2 and 3) at any follow-up visit. Growth of the infants in both groups were in alignment with WHO growth standards (mean Z-score within ±1 SD).

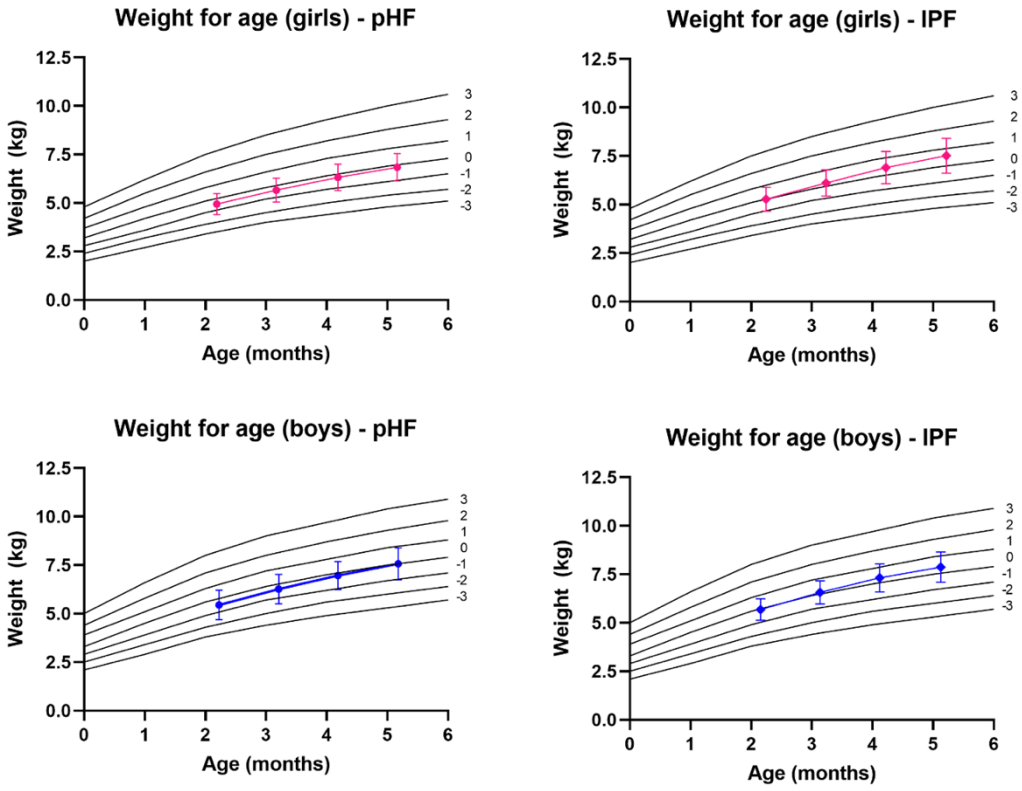

Figure 4-4. Mean weight-for-age Z-scores for girls and boys during the intervention according to study group.

pHF: Partially hydrolyzed whey infant formula; IPF: Intact protein formula

Associations of GI comfort parameters with formula intake and growth parameters

Formula consumption of infants in both groups at all three follow-up measurements are reported in Supplementary file 4. The average baseline weight of the infants in both groups was above 5 kg, therefore the projected formula consumption, according to the feeding table, would be

approximately 1,000 mL/day. With the exception of the fourth visit for the IPF group, all reported average formula intakes were slightly below 1,000 mL.

Analyses for associations between GI comfort parameters with milk intake showed that a higher milk intake appeared to be associated with smaller stools (r=-0.140, p=0.041) and less crying (r=-0.209, p=0.002) and fussiness (r=-0.149, p=0.028) in the pHF group. A higher milk intake was also positively associated with a higher frequency of (r=0.149, p=0.028) and more formed stools (r=0.157, p=0.021) in both groups, although in the IPF group, this was only found for higher milk intake per kg body weight (r=0.171, p=0.013 and r=0.172, p=0.013, respectively). Additionally, a higher milk intake per kg body weight was positively associated with vomiting (r=0.172, p=0.013), gassiness (r=0.166, p=0.016) and overall IGSQ scores (r=0.202, p=0.003) in the IPF group.

Associations between GI comfort and growth parameters were observed only in the pHF group. Overall, no correlations were observed between growth and IGSQ scores, however there were correlations with several AISS parameters (Supplementary file 5). A negative correlation was noted between mean stool volume and infant BMI (r=-0.213, p=0.002), indicating that bigger infants produced smaller stools. On the other hand, a positive correlation was observed between mean stool consistency and infant BMI (r=0.208, p=0.002), with bigger infants producing harder stools.

Discussion

The present analysis of tertiary outcomes showed that infants receiving a commercially available minimally processed infant formula with intact protein displayed some differences in stool parameters compared to those who received a partially hydrolysed whey-based infant formula. Overall, both groups experienced good GI comfort. We further show that infants in both groups showed similar growth trajectories in accordance with the WHO growth references.

The current study investigated how protein modification affects parameters related to the digestive comfort of healthy infants. Previous studies have shown some beneficial effects of formulas containing pHF on functional GI manifestations including constipation (Huang et al., 2021; Picaud et al., 2020; Savino et al., 2005; Slavin, 2013). The latter is reported to commonly occur in children up to 48 months of age (Havlicekova et al., 2016). To the best of our knowledge, in all of the reported cases, test formulas contained additional ingredients that are known to be linked with the investigated benefits.

For example, in a study conducted by Savino et al., apart from partially hydrolysed whey protein, the test formula also contained a prebiotic mixture of galacto- and fructo-oligosaccharides with a high beta-palmitic acid content (Savino et al., 2005). Beta-palmitate, among many other benefits, has also been shown to improve stool consistency, increase stool frequency and reduce crying time (Havlicekova et al., 2016; Litmanovitz et al., 2014). Additionally, prebiotic fibres such as galacto- and fructo-oligosaccharides, are also known to improve stool frequency and consistency (Slavin, 2013). The same formula was also shown to reduce the frequency of regurgitation; however, this effect may be attributed to the starch in the test formula, which is a well-known thickener, thus, in turn, having a significant effect on regurgitation.

Another study by Huang et al. reported that pHF containing low lactose and probiotics improves GI functions in infants with mild GI disorders (Huang et al., 2021). The addition of probiotics in the formula, especially Bifidobacteria that are abundant in breastmilk, is postulated to promote a colonic environment that contributes to GI health benefits. In our study, the two formulas evaluated had quite a comparable composition except for the protein being either partially hydrolysed or intact and the concentration of Galacto-oligosaccharides (GOS). Studies reported that a higher concentration of GOS has been associated with softer stools (Huppertz and Chia, 2021).

The higher stool volume and lower consistency reported in this study for the pHF does not seem to be linked with overall GI comfort of the healthy infants participating in this study. This may be due to the fact that, despite the significant difference between both groups, both scores are relatively low placing between watery and soft categories. In addition, the outcome is to answer the tertiary objectives which may not be supported by the sample size calculation. However, it still cannot be excluded that partially hydrolysed protein with GOS may offer benefits for infants experiencing constipation.

Gastrointestinal comfort has been linked to low levels of glycation and low levels of casein mineralization present in infant formula. A recent study found that levels of blocked lysine in four different commercially available infant formulas ranged from 9 to 20% (Sheng et al., 2020). The study also found that the occurrence of GI symptoms and crying time at night was lower among infants fed with minimally processed formula (low levels of blocked lysine, < 10%). Low casein mineralization is also important in infant nutrition to allow for easier digestion in the infant stomach, considering its limitations in enzymatic activity, motility and stomach capacity (Den Hertog et al., 2012). The processes in the manufacturing of formula are therefore important

owing to their impact on levels of glycation and casein mineralization. In the current study, the minimally processed high-quality IPF, with low glycation (<10 %) and low casein mineralization (≤7.5 mmol micellar Ca/10g casein), has been shown to provide good effects on GI comfort.

There was no difference observed in the defecation frequency between both groups. The 1.4 to 1.5 daily defecation rate is similar to that reported by Hertog et al. from a cohort of 600 healthy babies at 3 months of age (1.88 for breastfed, 1.37 for bottle-fed and 1.54 for mixed feeding) (Den Hertog et al., 2012). The frequency of defecation was measured from approximately 3 months of age, which may explain why we have not observed age-related decrease, as the biggest drop happens in the first months of age, and is also more characteristic of breast-fed babies.

In the same cohort, the authors reported that the majority of infants fed with IPF had green-coloured stools (Den Hertog et al., 2012). In our study, green-coloured stool was characteristic for the babies fed with pHF, while those fed with IPF had mostly yellow-coloured stools at all measured time points. The yellow colour for this particular formula was already reported in a previous study in a group of healthy Chinese infants (Sheng et al., 2020). Green colour, on the other hand, is often anecdotally reported for pHF, with the colour likely to originate from the bile that is excreted because of the rapid transfer through the intestine of the pre-digested protein in the pHF. Green stools can also be seen from breastfed infants if they do not finish nursing on one breast and thus do not ingest sufficient fat, which is most abundant in the hindmilk. However, given that the fat component is not modified in pHF and that the frequency of defecation is not increased, this hypothesis may not be valid.

The infants in this study also showed healthy weight gain as per the WHO growth standards without any evidence of excessive milk intake. However, even within this group of healthy growing infants, we have observed associations between higher milk intake per kg of body weight and comfort-related parameters. In lieu of increased prevalence of obesity in later life, having optimal milk intake during early childhood is preferable.

It is well recognized in literature and clinical practice that overfeeding is frequently linked with regurgitation (Benninga et al., 2016). In our data, we also see that higher milk intakes affect gassiness and overall IGSQ scores. Additionally, a small subset of infants in this study who had healthy Z-scores at birth, no longer had healthy Z-scores at baseline. While the change in Z-scores indicated some growth issues among these infants, the results showed that most of them had

normal growth trajectories while consuming either study formula. Notably, also, the infants who were healthy at baseline continued to show healthy growth trajectories.

Conclusions

Findings from this study indicate that despite some differences in stool consistency, volume, colour, and gassiness, the overall digestive comfort reported was comparable between the two groups of infants fed with either minimally processed IPF or pHF. Both formulas promote good GI comfort and optimal infant growth in accordance with WHO growth references.

Supplementary file 1.

Table S. 4-7. Composition of the study formulas (per 100 mL).

	pHF	**IPF**
Energy (kcal)	66	66
Intact protein (g)		1.4
Casein		0.57
Whey		0.85
Whey protein hydrolysate (g)	1.6	
Fat (g)	3.5	3.5
DHA (mg)	6.9	6.9
AA (mg)	6.9	6.9
Carbohydrates	7.0	7.0
GOS (g)	0.2	0.4
Ca (mg)	50	56
P (mg)	30	31
Na (mg)	20	23
Fe (mg)	0.78	0.77
Vitamin D (µg)	1.2	1.1

pHF: Partially hydrolyzed whey infant formula; IPF: Intact protein formula; AA: Arachidonic acid; DHA: Docosahexaenoic acid; GOS: Galacto-oligosaccharides; Ca: Calcium; P: Phosphorus; Na: Sodium; Fe: Iron.

Supplementary file 2.

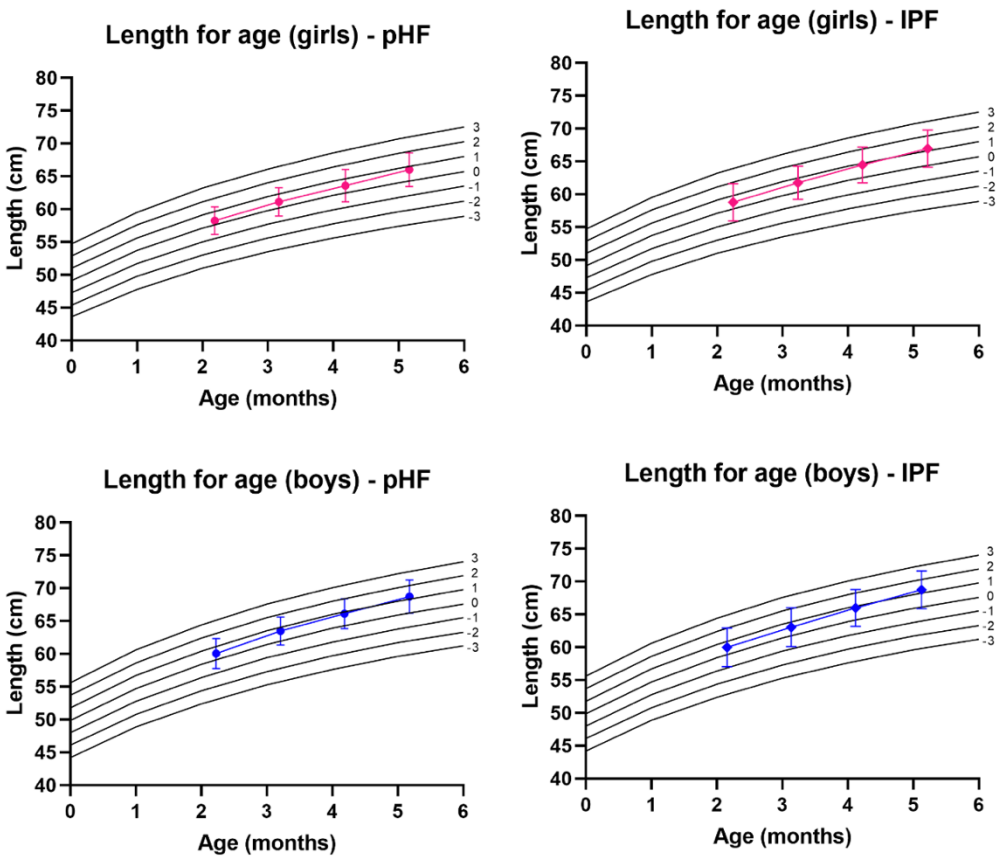

Figure S. 4-2. Mean length-for-age Z-scores for girls and boys during the intervention according to study group.

pHF: Partially hydrolyzed whey infant formula; IPF: Intact protein formula

Supplementary file 3.

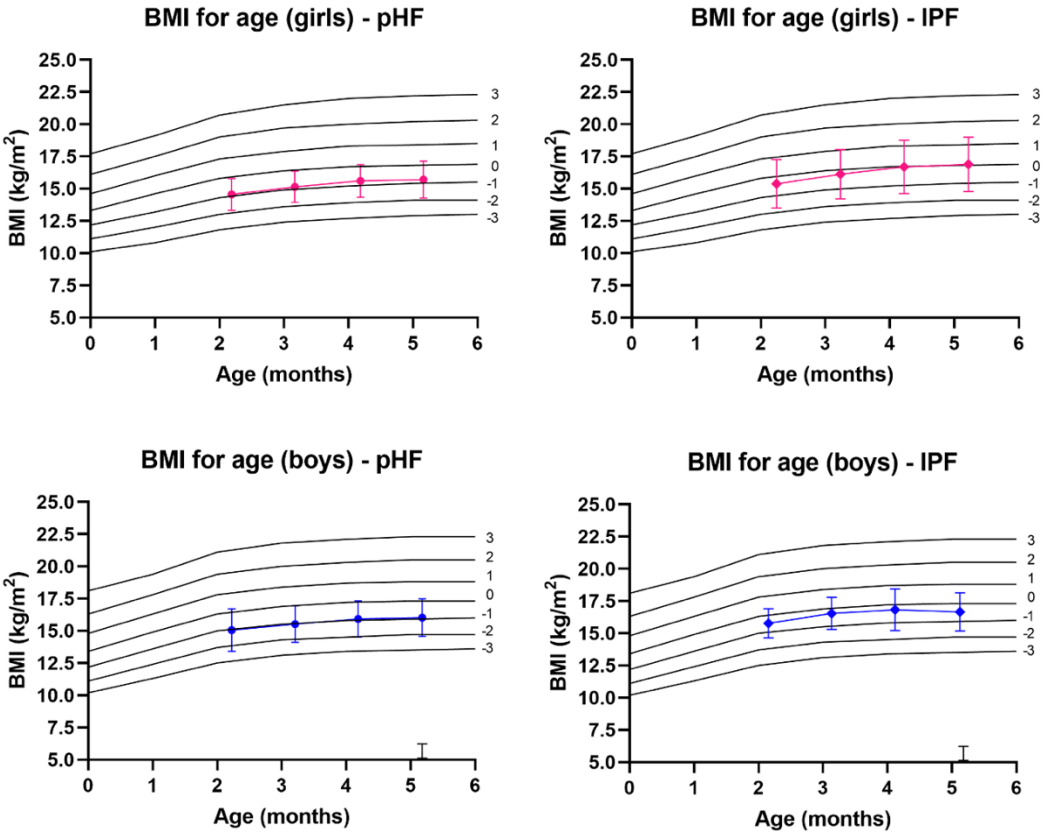

Figure S. 4-3. Mean BMI-for-age Z-scores for girls and boys during the intervention according to study group.

pHF: Partially hydrolyzed whey infant formula; IPF: Intact protein formula; BMI: Body mass index

Supplementary file 4.

Table S. 4-8. Mean daily formula intake[1] during the intervention in the PP population.

Formula intake	Visit	pHF (N=72)	IPF (N=70)
Average daily intake, mL/d, mean (SD)	2	842.06 (165.82)	918.14 (152.06)
	3	894.05 (218.26)	993.14 (163.86)
	4	942.01 (195.65)	1016.74 (155.54)
Average daily intake per kg body weight, mL/kg/d, mean (SD)	2	143.13 (27.86)	145.12 (24.36)
	3	135.19 (28.91)	140.10 (23.37)
	4	131.84 (24.68)	132.76 (20.44)

pHF: Partially hydrolyzed whey infant formula; IPF: Intact protein formula; N: Number of subjects in analysis population; SD: Standard deviation.

[1]*Reported in the 7-day milk intake diary preceding each visit*

Supplementary file 5.

Table S. 4-9. Correlations between GI comfort and growth parameters in the pHF group.

Parameter	**Weight**	**WFL**	**BMI**
	r, p	**r, p**	**r, p**
IGSQ	0.007, 0.918	0.048, 0.477	0.122, 0.073
AISS Stool frequency	-0.054, 0.430	-0.011, 0.874	-0.032, 0.637
AISS Stool volume	-0.139*, 0.040	-0.192**, 0.005	-0.213**, 0.002
AISS Stool colour	-0.014, 0.835	-0.036, 0.597	-0.081, 0.231
AISS Stool consistency	0.149*, 0.028	0.173*, 0.010	0.208**, 0.002

GI: Gastrointestinal; pHF: Partially hydrolyzed whey infant formula; WFL: Weight for length; BMI: Body mass index; IGSQ: Infant Gastrointestinal Symptoms Questionnaire; AISS: Amsterdam Infant Stool Scale.

** Correlation is significant at the 0.05 level (2-tailed); ** Correlation is significant at the 0.01 level (2-tailed); n=218 for all measurements.*

5. DISCUSSION

The current Thesis aimed to (a) investigate the effect of bovine milk fat used in the fat blend of infant formulas, on stool fatty acid soaps, calcium excretion and stool characteristics of healthy term infants; (b) investigate the effects of a partially hydrolysed whey infant formula on growth in healthy term infants as compared to a standard infant formula with intact protein; and (c) investigate the effects of the same partially hydrolysed whey infant formula on digestive comfort parameters of healthy term infants compared to the intact protein formula, as well as to assess links of corresponding growth data with gastrointestinal comfort.

Two randomized clinical trials were conducted with healthy term, exclusively formula-fed infants: the Little Panda study, examining point (a) above and the SHIFT study, examining points (b) and (c) above.

Little Panda study actually comprised of two double-blind, randomised cross-over trials, conducted in parallel with healthy infants aged 9–14 weeks at baseline. In total, 16 and 17 infants completed each cross-over study, respectively. To our knowledge, this is the first study to date directly comparing infant formulas with fat blends from bovine milk in two different dosages and a traditional, standard formula with a 100% vegetable fat blend. Significant favourable effects were observed in stool palmitic acid-soaps and total fatty acid-soaps for both milk fat groups compared to the vegetable fat group, while no significant effects were observed for stool free palmitic acid and total fatty acids. Calcium excretion in the faeces was significantly lower in both milk fat groups compared to the vegetable fat group. Furthermore, the mean stool consistency score was significantly lower in the 50% milk fat group compared to the vegetable fat group, while no such difference was observed for the 20% milk fat group.

Despite the differences observed in the palmitic acid -soaps and total fatty acid-soaps excreted in the faeces among the milk fat and the vegetable fat formulas, a potential better fat and caloric absorption that could affect infants' growth and development cannot be demonstrated by the current study. As this was a cross-over study design with a short intervention period (two periods of two weeks each), it would be interesting to investigate growth indices prospectively using two treatment arms (milk fat formula vs. vegetable fat formula), as well as a breastfeeding reference group.

In the same context, a number of studies have demonstrated the beneficial effect of high SN-2 formulas on bone mass and bone strength/quality (bone mineral density determined by dual-

energy x-ray absorptiometry or bone speed of sound by quantitative ultrasound) (Kennedy et al., 1999; Litmanovitz et al., 2013). Thus, it would be interesting to explore the potential beneficial effects of the milk fat formulas on these indices, since faecal calcium excretion was found to be significantly lower in both milk fat groups of the current study compared to the vegetable fat group. Further research is also needed to determine the biological effect of reduced calcium excretion on infants both in the short-term (study duration) but also in the long run (track into childhood).

With regards to stool consistency, it is known that breastfed infants have more frequent and runny or loose soft stools than formula-fed infants (Quinlan et al., 1995; Weaver et al., 1988). In particular, formula-fed infants have less frequent bowel movements and firmer stools that may, in some cases, be difficult to pass, thus leading to discomfort. These changes in stool patterns are often perceived as abnormal by parents and are hence a common source of parental distress and a frequent cause of consultation to health care providers. Therefore, it would also be interesting to explore the potential favourable effects of the milk fat formulas on stool consistency in combination with the potential changes on gastrointestinal symptoms and parental concerns.

Last but not least, the vast majority of the existing studies have used synthetic triacylglycerols to increase the SN-2 content of the infant formula, while this is the first study to evaluate the effects of high SN-2 content derived from milk fat blends. So, it would be interesting to compare the effects of a milk fat formula vs. a synthetic formula on fat and calcium absorption, as well as on stool characteristics and bone mass indices. Additionally, as it has been suggested that soap formation may also be influenced by factors independent of triacylglycerol structure, such as the presence of the prebiotic oligofructose (Nowacki et al., 2014; Yao et al., 2014), the potential additional benefit of supplementing milk fat infant formula with oligofructose could be further investigated.

SHIFT study was a double-blind, non-inferiority, randomised trial conducted with 163 healthy infants aged 9–14 weeks at baseline. In total, 142 infants completed the study. No differences in daily weight gain were observed between the partially hydrolysed whey infant formula and the standard infant formula with intact protein during the three-month intervention period. Furthermore, no differences were observed between the two groups at any time point in other growth parameters examined, i.e. infants' weight, length, head circumference, BMI, and their respective Z-scores, all being within the normal range of the WHO growth standards. Despite

some differences in stool consistency, volume, colour, and gassiness, the overall digestive comfort reported was also found to be comparable between the two groups of formula fed infants. Therefore, the current findings suggest that both infant formulas promote good gastrointestinal comfort and support normal growth in accordance with the WHO standards.

6. MAIN THESIS CONTRIBUTIONS

Manuscript 1: Palmitic acid is one of the most abundant saturated fatty acids in human milk, with approximately 70% structurally positioned at the SN-2 position of triacylglycerol molecules, which is particularly well-absorbed and exerts beneficial effects on fat and calcium absorption and stool consistency in healthy infants. Most infant formulas use a vegetable fat blend as a source of fat, which has a lower total palmitic acid content and a lower percentage of palmitic acid at the SN-2 position compared to human milk. The use of milk fat, a natural source of SN-2-palmitate, in infant formula reduced stool palmitic acid soaps and calcium excretion in healthy term infants compared to a vegetable fat blend formula in Little Panda study. Therefore, milk fat formula is suggested to improve gastrointestinal outcomes in healthy term infants.

Manuscript 2: Hydrolysed protein formulas are mainly developed for allergy prevention and management. The different types of hydrolysed protein formulas and different brands vary in their composition which may influence formula consumption and growth patterns. In the SHIFT study, growth trajectories of healthy term formula-fed infants were within the normal range based on WHO growth standards in both the partially hydrolysed whey-based infant formula and the standard infant formula with intact protein. Therefore, partially hydrolysed whey-based infant formula supports normal growth in healthy term infants.

Manuscript 3: Infant formula manufacturing processes and composition have an effect on the digestibility of the formula. Partially hydrolysed formula may be gentler on the digestive system as hydrolysis enzymatically digests protein into smaller peptides. Tertiary analysis of the SHIFT study showed that infants receiving a commercially available minimally processed infant formula with intact protein displayed some differences in stool parameters (particularly stool consistency, volume, colour, and gassiness) compared to those who received the partially hydrolysed whey-based infant formula; however, the overall digestive comfort reported was comparable between the two groups and both formulas are suggested to promote good gastrointestinal comfort.

7. CONCLUSIONS

Manuscript 1: The findings of Little Panda study showed that the use of bovine milk fat in the development of infant formula, leading to higher SN-2 palmitate content, results in lower levels of palmitic acid soaps, total fatty acid soaps and calcium in stool samples of healthy term infants compared to a traditional vegetable fat formula. High SN-2 milk fat formula can have additional favourable effect on infants' stool consistency. These findings suggest that fat, calories and calcium are plausibly more efficiently absorbed when a milk fat formula is used. Future research could further explore the clinical benefits of the present outcomes on gut comfort, growth and development of healthy infants.

Manuscript 2: According to new European Commission regulations, applying to hydrolysate-based formulas from 2021 onwards, each specific hydrolysate-based formula needs to be evaluated for their safety and suitability by the European Food Safety Authority (EFSA). The SHIFT study demonstrated that weight gain as well as other growth outcomes did not differ between infants consuming the partially hydrolysed whey-based infant formula and those consuming a standard intact protein-based formula. All Z-score indices obtained were within the normal range of WHO growth standards. Based on these results, it can be concluded that the infant formula with partially hydrolysed whey protein supports normal growth in healthy term infants.

Manuscript 3: Tertiary analysis of the SHIFT study investigated how protein modification affects parameters related to the digestive comfort of healthy infants. The present findings showed that despite some differences in stool consistency, volume, colour, and gassiness, the overall digestive comfort reported was comparable between the two groups of infants fed with either a minimally processed intact protein-based formula or a partially hydrolysed whey-based infant formula. Both formulas promote good gastrointestinal comfort and optimal infant growth in accordance with the WHO growth standards.

REFERENCES

Alexander, D.D., Cabana, M.D., 2010. Partially hydrolyzed 100% whey protein infant formula and reduced risk of atopic dermatitis: a meta-analysis. J Pediatr Gastroenterol Nutr 50, 422–430. https://doi.org/10.1097/MPG.0B013E3181CEA52B

American Academy of Pediatrics Task Force, 2015. Clinical Testing of Infant Formulas With Respect to Nutritional Suitability for Term Infants [WWW Document]. URL http://wayback.archive-it.org/7993/20170722090324/https://www.fda.gov/Food/GuidanceRegulation/GuidanceDocumentsRegulatoryInformation/InfantFormula/ucm170649.htm (accessed 4.4.23).

Andreas, N.J., Kampmann, B., Mehring Le-Doare, K., 2015. Human breast milk: A review on its composition and bioactivity. Early Hum Dev. https://doi.org/10.1016/j.earlhumdev.2015.08.013

Baird, J., Fisher, D., Lucas, P., Kleijnen, J., Roberts, H., Law, C., 2005. Being big or growing fast: systematic review of size and growth in infancy and later obesity. BMJ 331, 929–931. https://doi.org/10.1136/BMJ.38586.411273.E0

Bar-Yoseph, F., Lifshitz, Y., Cohen, T., Malard, P., Xu, C., 2016. SN2-palmitate reduces fatty acid excretion in Chinese formula-fed infants. J Pediatr Gastroenterol Nutr 62, 341–347. https://doi.org/10.1097/MPG.0000000000000971

Béghin, L., Marchandise, X., Lien, E., Bricout, M., Bernet, J.P., Lienhardt, J.F., Jeannerot, F., Menet, V., Requillart, J.C., Marx, J., De Groot, N., Jaeger, J., Steenhout, P., Turck, D., 2019. Growth, stool consistency and bone mineral content in healthy term infants fed sn-2-palmitate-enriched starter infant formula: A randomized, double-blind, multicentre clinical trial. Clinical Nutrition 38, 1023–1030. https://doi.org/10.1016/j.clnu.2018.05.015

Bekkali, N., Hamers, S.L., Reitsma, J.B., Van Toledo, L., Benninga, M.A., 2009. Infant Stool Form Scale: Development and Results. Journal of Pediatrics 154. https://doi.org/10.1016/j.jpeds.2008.10.010

Benninga, M.A., Nurko, S., Faure, C., Hyman, P.E., St James Roberts, I., Schechter, N.L., 2016. Childhood Functional Gastrointestinal Disorders: Neonate/Toddler. Gastroenterology 150, 1443-1455.e2. https://doi.org/10.1053/J.GASTRO.2016.02.016

Berger, P.K., Plows, J.F., Demerath, E.W., Fields, D.A., 2020. Carbohydrate composition in breast milk and its effect on infant health. Curr Opin Clin Nutr Metab Care. https://doi.org/10.1097/MCO.0000000000000658

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

Bhatia, J., Shamir, R., Vandenplas, Y. (Eds.), 2016. Protein in Neonatal and Infant Nutrition: Recent Updates, Nestlé Nutrition Institute Workshop Series. S. Karger AG. https://doi.org/10.1159/ISBN.978-3-318-05483-5

Billeaud, C., Guillet, J., Sandler, B., 1990. Gastric emptying in infants with or without gastro-oesophageal reflux according to the type of milk. Eur J Clin Nutr 44, 577–83.

Bongers, M.E.J., De Lorijn, F., Reitsma, J.B., Groeneweg, M., Taminiau, J.A.J.M., Benninga, M.A., 2007. The clinical effect of a new infant formula in term infants with constipation: a double-blind, randomized cross-over trial. Nutr J 6. https://doi.org/10.1186/1475-2891-6-8

Carnielli, V.P., Luijendijk, I., Van Goudoever, J.B., Sulkers, E.J., Boerlage, A.A., Degenhart, H.J., Sauer, P.J., 1995. Feeding premature newborn infants palmitic acid in amounts and stereoisomeric position similar to that of human milk: effects on fat and mineral balance. Am J Clin Nutr 61, 1037–1042.

Carnielli, V.P.;, Luijendijk, I.H.T.;, Van Goudoever, J.B.;, Sulkers, E.J.;, Boerlage, A.A.;, Degenhart, H.J.;, Sauer, P.J.J., 1996. Structural Position and Amount of Palmitic Acid in Infant Formulas: Effects on Fat, Fatty Acid, and Mineral Balance. J Pediatr Gastroenterol Nutr 23, 553–560.

Chassard, C., de Wouters, T., Lacroix, C., 2014. Probiotics tailored to the infant: a window of opportunity. Curr Opin Biotechnol 26, 141–147. https://doi.org/10.1016/J.COPBIO.2013.12.012

Chomtho, S., Wells, J.C.K., Williams, J.E., Davies, P.S.W., Lucas, A., Fewtrell, M.S., 2008. Infant growth and later body composition: evidence from the 4-component model. Am J Clin Nutr 87, 1776–1784. https://doi.org/10.1093/AJCN/87.6.1776

Coppa, G. V., Gabrielli, O., Pierani, P., Catassi, C., Carlucci, A., Giorgi, P.L., 1993. Changes in Carbohydrate Composition in Human Milk Over 4 Months of Lactation. Pediatrics 91, 637–641. https://doi.org/10.1542/PEDS.91.3.637

Corvaglia, L., Mariani, E., Aceti, A., Galletti, S., Faldella, G., 2013. Extensively hydrolyzed protein formula reduces acid gastro-esophageal reflux in symptomatic preterm infants. Early Hum Dev 89, 453–455. https://doi.org/10.1016/J.EARLHUMDEV.2013.04.003

Delplanque, B., Gibson, R., Koletzko, B., Lapillonne, A., Strandvik, B., 2015. Lipid Quality in Infant Nutrition: Current Knowledge and Future Opportunities. J Pediatr Gastroenterol Nutr 61, 8–17. https://doi.org/10.1097/MPG.0000000000000818

Den Hertog, J., Van Leengoed, E., Kolk, F., Van Den Broek, L., Kramer, E., Bakker, E.J., Bakker-Van Gijssel, E., Bulk, A., Kneepkens, F., Benninga, M.A., 2012. The defecation pattern of healthy term infants up to the age of 3 months. Arch Dis Child Fetal Neonatal Ed 97. https://doi.org/10.1136/ARCHDISCHILD-2011-300539

Dewey, K.G., 2001. Nutrition, growth, and complementary feeding of the breastfed infant. Pediatr Clin North Am 48, 87–104.

Diepvens, K., Häberer, D., Westerterp-Plantenga, M., 2008. Different proteins and biopeptides differently affect satiety and anorexigenic/orexigenic hormones in healthy humans. Int J Obes (Lond) 32, 510–518. https://doi.org/10.1038/SJ.IJO.0803758

Diether, N.E., Willing, B.P., 2019. Microbial Fermentation of Dietary Protein: An Important Factor in Diet–Microbe–Host Interaction. Microorganisms 7. https://doi.org/10.3390/MICROORGANISMS7010019

Drapala, K.P., Auty, M.A.E., Mulvihill, D.M., O'Mahony, J.A., 2016. Improving thermal stability of hydrolysed whey protein-based infant formula emulsions by protein-carbohydrate conjugation. Food Res Int 88, 42–51. https://doi.org/10.1016/J.FOODRES.2016.01.028

Eidelman, A.I., Schanler, R.J., Johnston, M., Landers, S., Noble, L., Szucs, K., Viehmann, L., 2012. Breastfeeding and the Use of Human Milk. Pediatrics 129, e827–e841. https://doi.org/10.1542/PEDS.2011-3552

European Commission, 2015. Commission Delegated Regulation (EU) 2016/127.

Exl, B.M., Deland, U., Secretin, M.C., Preysch, U., Wall, M., Shmerling, D.H., 2000. Improved general health status in an unselected infant population following an allergen-reduced dietary intervention programme: the ZUFF-STUDY-PROGRAMME. Part II: infant growth and health status to age 6 months. ZUg-FrauenFeld. Eur J Nutr 39, 145–56. https://doi.org/10.1007/s003940070018

Florendo, K.N., Bellflower, B., van Zwol, A., Cooke, R.J., 2009. Growth in preterm infants fed either a partially hydrolyzed whey or an intact casein/whey preterm infant formula. Journal of Perinatology 29, 106–111. https://doi.org/10.1038/jp.2008.124

Foltz, M., Ansems, P., Schwarz, J., Tasker, M.C., Lourbakos, A., Gerhardt, C.C., 2008. Protein hydrolysates induce CCK release from enteroendocrine cells and act as partial agonists of the CCK1 receptor. J Agric Food Chem 56, 837–843. https://doi.org/10.1021/JF072611H

Fragkou, P.C., Karaviti, D., Zemlin, M., Skevaki, C., 2021. Impact of Early Life Nutrition on Children's Immune System and Noncommunicable Diseases Through Its Effects on the

Bacterial Microbiome, Virome and Mycobiome. Front Immunol 12. https://doi.org/10.3389/FIMMU.2021.644269

Fritsché, R., Pahud, J.J., Pecquet, S., Pfeifer, A., 1997. Induction of systemic immunologic tolerance to beta-lactoglobulin by oral administration of a whey protein hydrolysate. J Allergy Clin Immunol 100, 266–273. https://doi.org/10.1016/S0091-6749(97)70235-5

Ghanma, A., Puttemans, K., Deneyer, M., Benninga, M.A., Vandenplas, Y., 2014. Amsterdam infant stool scale is more useful for assessing children who have not been toilet trained than Bristol stool scale. Acta Paediatrica, International Journal of Paediatrics 103. https://doi.org/10.1111/apa.12422

Green Corkins, K., Shurley, T., 2016. What's in the Bottle? A Review of Infant Formulas. Nutr Clin Pract 31, 723–729. https://doi.org/10.1177/0884533616669362

Halken, S., Hansen, K.S., Jacobsen, H.P., Estmann, A., Christensen, A.E.F., Hansen, L.G., Kier, S.R., Lassen, K., Lintrup, M., Mortensen, S., Ibsen, K.K., Østerballe, O., Høst, A., 2000. Comparison of a partially hydrolyzed infant formula with two extensively hydrolyzed formulas for allergy prevention: a prospective, randomized study. Pediatr Allergy Immunol 11, 149–161. https://doi.org/10.1034/J.1399-3038.2000.00081.X

Haschke, F., Binder, C., Huber-Dangl, M., Haiden, N., 2019. Early-Life Nutrition, Growth Trajectories, and Long-Term Outcome. Nestle Nutr Inst Workshop Ser 90, 107–120. https://doi.org/10.1159/000490299

Havlicekova, Z., Jesenak, M., Banovcin, P., Kuchta, M., 2016. Beta-palmitate - A natural component of human milk in supplemental milk formulas. Nutr J 15. https://doi.org/10.1186/s12937-016-0145-1

Heine, R.G., 2008. Allergic gastrointestinal motility disorders in infancy and early childhood. Pediatr Allergy Immunol 19, 383–391. https://doi.org/10.1111/J.1399-3038.2008.00785.X

Hernández-Ledesma, B., García-Nebot, M.J., Fernández-Tomé, S., Amigo, L., Recio, I., 2014. Dairy protein hydrolysates: Peptides for health benefits. Int Dairy J 38, 82–100. https://doi.org/10.1016/J.IDAIRYJ.2013.11.004

Host, A., Halken, S., 2014. Cow's milk allergy: where have we come from and where are we going? Endocr Metab Immune Disord Drug Targets 14, 2–8. https://doi.org/10.2174/1871530314666140121142900

Høst, A., Halken, S., 2005. Primary prevention of food allergy in infants who are at risk. Curr Opin Allergy Clin Immunol 5, 255–259. https://doi.org/10.1097/01.ALL.0000168791.89829.2A

Høst, A., Koletzko, B., Dreborg, S., Muraro, A., Wahn, U., Aggett, P., Bresson, J.L., Hernell, O., Lafeber, H., Michaelsen, K.F., Micheli, J.L., Rigo, J., Weaver, L., Heymans, H., Strobel, S., Vandenplas, Y., 1999. Dietary products used in infants for treatment and prevention of food allergy. Joint Statement of the European Society for Paediatric Allergology and Clinical Immunology (ESPACI) Committee on Hypoallergenic Formulas and the European Society for Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) Committee on Nutrition. Arch Dis Child 81, 80–84. https://doi.org/10.1136/ADC.81.1.80

Huang, Y., Zhou, Y., Li, H., Chen, Y., Mu, Y., Yuan, A., Yang, Y., Liu, J., 2021. The effects of a partially hydrolyzed formula with low lactose and probiotics on mild gastrointestinal disorders of infants: A single-armed clinical trial. Nutrients 13. https://doi.org/10.3390/nu13103371

Huppertz, T., Chia, L.W., 2021. Milk protein coagulation under gastric conditions: A review. Int Dairy J 113, 104882. https://doi.org/10.1016/J.IDAIRYJ.2020.104882

Innis, S.M., 2014. Impact of maternal diet on human milk composition and neurological development of infants,,. Am J Clin Nutr 99, 734S-741S. https://doi.org/10.3945/AJCN.113.072595

Innis, S.M., 2011. Dietary triacylglycerol structure and its role in infant nutrition. Advances in Nutrition. https://doi.org/10.3945/an.111.000448

Jiang, Y.J., 2014. Human Milk Biochemistry and Infant Formula Manufacturing Technology, Human Milk Biochemistry and Infant Formula Manufacturing Technology.

Karaglani, E., Thijs-verhoeven, I., Gros, M., Chairistanidou, C., Zervas, G., Filoilia, C., Kampani, T.M., Miligkos, V., Matiatou, M., Valaveri, S., Sakellariou, A., Babilis, G., Bos, R., Manios, Y., 2020. A partially hydrolyzed whey infant formula supports appropriate growth: A randomized controlled non-inferiority trial. Nutrients 12, 1–11. https://doi.org/10.3390/nu12103056

Katz, Y., Rajuan, N., Goldberg, M.R., Eisenberg, E., Heyman, E., Cohen, A., Leshno, M., 2010. Early exposure to cow's milk protein is protective against IgE-mediated cow's milk protein allergy. J Allergy Clin Immunol 126. https://doi.org/10.1016/J.JACI.2010.04.020

Kennedy, K., Fewtrell, M., Morley, R., Abbott, R., Quinlan, P.T., Wells, J.C., Bindels, J.G., Lucas, A., 1999. Double-blind, randomized trial of a synthetic triacylglycerol in formula-fed term infants: effects on stool biochemistry, stool characteristics, and bone mineralization. American Journal of Clinical Nutrition 70, 920–927.

Koletzko, B., Agostoni, C., Bergmann, R., Ritzenthaler, K., Shamir, R., 2011. Physiological aspects of human milk lipids and implications for infant feeding: a workshop report. Acta Paediatr 100, 1405–1415. https://doi.org/10.1111/J.1651-2227.2011.02343.X

Koletzko, B., Mrotzek, M., Bremer, H.J., 1988. Fatty acid composition of mature human milk in Germany. Am J Clin Nutr 47, 954–959. https://doi.org/10.1093/AJCN/47.6.954

Koletzko, B., Rodriguez-Palmero, M., Demmelmair, H., Fidler, N., Jensen, R., Sauerwald, T., 2001. Physiological aspects of human milk lipids. Early Hum Dev 65, S3–S18.

Koletzko, S., Niggemann, B., Arato, A., Dias, J.A., Heuschkel, R., Husby, S., Mearin, M.L., Papadopoulou, A., Ruemmele, F.M., Staiano, A., Schäppi, M.G., Vandenplas, Y., 2012. Diagnostic approach and management of cow's-milk protein allergy in infants and children: ESPGHAN GI Committee practical guidelines. J Pediatr Gastroenterol Nutr 55, 221–229. https://doi.org/10.1097/MPG.0B013E31825C9482

Koopman, R., Crombach, N., Gijsen, A.P., Walrand, S., Fauquant, J., Kies, A.K., Lemosquet, S., Saris, W.H.M., Boirie, Y., Van Loon, L.J.C., 2009. Ingestion of a protein hydrolysate is accompanied by an accelerated in vivo digestion and absorption rate when compared with its intact protein. Am J Clin Nutr 90, 106–115. https://doi.org/10.3945/AJCN.2009.27474

L. Barrera, E., Ramirez-Farias, C., Marriage, B.J., 2021. Nutritional Management of Cow's Milk Allergy in Infants: A Comparison of DRACMA, ESPGHAN, and AAP Guidelines. Open Nutr J 15, 1–9. https://doi.org/10.2174/1874288202115010001

Lindquist, S., Hernell, O., 2010. Lipid digestion and absorption in early life: An update. Curr Opin Clin Nutr Metab Care. https://doi.org/10.1097/MCO.0b013e328337bbf0

Litmanovitz, I., Bar-Yoseph, F., Lifshitz, Y., Davidson, K., Eliakim, A., Regev, R.H., Nemet, D., 2014. Reduced crying in term infants fed high beta-palmitate formula: A double-blind randomized clinical trial. BMC Pediatr 14. https://doi.org/10.1186/1471-2431-14-152

Litmanovitz, I., Davidson, K., Eliakim, A., Regev, R.H., Dolfin, T., Arnon, S., Bar-Yoseph, F., Goren, A., Lifshitz, Y., Nemet, D., 2013. High beta-palmitate formula and bone strength in term infants: A randomized, double-blind, controlled trial. Calcif Tissue Int 92, 35–41. https://doi.org/10.1007/s00223-012-9664-8

Lonnerdal, B., 1986. Effects of maternal dietary intake on human milk composition. J Nutr 116, 499–513. https://doi.org/10.1093/JN/116.4.499

Lonnerdal, B., Forsum, E., Hambraeus, L., 1976. A longitudinal study of the protein, nitrogen, and lactose contents of human milk from Swedish well-nourished mothers. Am J Clin Nutr 29, 1127–1133. https://doi.org/10.1093/AJCN/29.10.1127

López-López, A., Castellote-Bargalló, A.I., Campoy-Folgoso, C., Rivero-Urgel, M., Tormo-Carnicé, R., Infante-Pina, D., López-Sabater, M.C., 2001. The influence of dietary palmitic acid triacylglyceride position on the fatty acid, calcium and magnesium contents of at term newborn faeces. Early Hum Dev 65 Suppl, S83–S94.

Lucas, A., Quinlan, P., Abrams, S., Ryan, S., Meah, S., Lucas, P.J., 1997. Randomised controlled trial of a synthetic triglyceride milk formula for preterm infants, Arch Dis Child.

Marie Straarup, E., Lauritzen, L., Faerk, J., HLy, C.-E., Michaelsen, K.F., 2006. The Stereospecific Triacylglycerol Structures and Fatty Acid Profiles of Human Milk and Infant Formulas, Journal of Pediatric Gastroenterology and Nutrition. Lippincott Williams & Wilkins.

Martin, C.R., Ling, P.R., Blackburn, G.L., 2016. Review of infant feeding: Key features of breast milk and infant formula. Nutrients. https://doi.org/10.3390/nu8050279

Mennella, J.A., Griffin, C.E., Beauchamp, G.K., 2004. Flavor programming during infancy. Pediatrics 113, 840–845. https://doi.org/10.1542/PEDS.113.4.840

Mennella, J.A., Ventura, A.K., Beauchamp, G.K., 2011. Differential growth patterns among healthy infants fed protein hydrolysate or cow-milk formulas. Pediatrics 127, 110–118. https://doi.org/10.1542/peds.2010-1675

Michaelsen, K.F., Skafte, L., Badsberg, J.H., Jørgensen, M., 1990. Variation in Macronutrients in Human Bank Milk: Influencing Factors and Implications for Human Milk Banking. J Pediatr Gastroenterol Nutr 11, 229–239.

Mihatsch, W.A., Högel, J., Pohlandt, F., 2001. Hydrolysed protein accelerates the gastrointestinal transport of formula in preterm infants. Acta Paediatr 90, 196–198. https://doi.org/10.1080/080352501300049442

Miles, E.A., Calder, P.C., 2017. The influence of the position of palmitate in infant formula triacylglycerols on health outcomes. Nutrition Research. https://doi.org/10.1016/j.nutres.2017.05.009

Musilova, S., Rada, V., Vlkova, E., Bunesova, V., 2014. Beneficial effects of human milk oligosaccharides on gut microbiota. Benef Microbes 5, 273–283. https://doi.org/10.3920/BM2013.0080

Nowacki, J., Lee, H.C., Lien, R., Cheng, S.W., Li, S.T., Yao, M., Northington, R., Jan, I., Mutungi, G., 2014. Stool fatty acid soaps, stool consistency and gastrointestinal tolerance in term infants fed infant formulas containing high sn-2 palmitate with or without oligofructose: A double-blind, randomized clinical trial. Nutr J 13. https://doi.org/10.1186/1475-2891-13-105

Nutten, S., 2016. Proteins, Peptides and Amino Acids: Role in Infant Nutrition. Nestle Nutr Inst Workshop Ser 86, 1–10. https://doi.org/10.1159/000442697

Pecquet, S., Bovetto, L., Maynard, F., Fritsché, R., 2000. Peptides obtained by tryptic hydrolysis of bovine beta-lactoglobulin induce specific oral tolerance in mice. J Allergy Clin Immunol 105, 514–521. https://doi.org/10.1067/MAI.2000.103049

Petit, V., Sandoz, L., Garcia-Rodenas, C.L., 2017. Importance of the regiospecific distribution of long-chain saturated fatty acids on gut comfort, fat and calcium absorption in infants. Prostaglandins Leukot Essent Fatty Acids. https://doi.org/10.1016/j.plefa.2017.05.007

Picaud, J.C., Pajek, B., Arciszewska, M., Tarczón, I., Escribano, J., Porcel, R., Adelt, T., Hassink, E., Rijnierse, A., Abrahamse-Berkeveld, M., Korczowski, B., 2020. An Infant Formula with Partially Hydrolyzed Whey Protein Supports Adequate Growth and Is Safe and Well-Tolerated in Healthy, Term Infants: A Randomized, Double-Blind, Equivalence Trial. Nutrients 12, 1–16. https://doi.org/10.3390/NU12072072

Puccio, G., Alliet, P., Cajozzo, C., Janssens, E., Corsello, G., Sprenger, N., Wernimont, S., Egli, D., Gosoniu, L., Steenhout, P., 2017. Effects of infant formula with human milk oligosaccharides on growth and morbidity: A randomized multicenter trial, in: Journal of Pediatric Gastroenterology and Nutrition. Lippincott Williams and Wilkins, pp. 624–631. https://doi.org/10.1097/MPG.0000000000001520

Quinlan, P., Lockton, S., Irwin, J., Lucas, A., 1995. The relationship between stool hardness and stool composition in breast- and formula-fed infants. J Pediatr Gastroenterol Nutr 20, 81–90. https://doi.org/10.1097/00005176-199501000-00014

Riley, A.W., Trabulsi, J., Yao, M., Bevans, K.B., Derusso, P.A., 2015. Validation of a parent report questionnaire. Clin Pediatr (Phila) 54, 1167–1174. https://doi.org/10.1177/0009922815574075

Rzehak, P., Sausenthaler, S., Koletzko, S., Reinhardt, D., Von Berg, A., Krämer, U., Berdel, D., Bollrath, C., Grübl, A., Bauer, C.P., Wichmann, H.E., Heinrich, J., 2009. Short- and long-term effects of feeding hydrolyzed protein infant formulas on growth at ≤6 y of age: Results from the German Infant Nutritional Intervention Study. American Journal of Clinical Nutrition 89, 1846–1856. https://doi.org/10.3945/ajcn.2008.27373

Savino, F., Maccario, S., Castagno, E., Cresi, F., Cavallo, F., Dalmasso, P., Fanaro, S., Oggero, R., Silvestro, L., 2005. Advances in the management of digestive problems during the first months of life. Acta Paediatr Suppl 94, 120–124. https://doi.org/10.1111/J.1651-2227.2005.TB02167.X

Savino, F., Palumeri, E., Castagno, E., Cresi, F., Dalmasso, P., Cavallo, F., Oggero, R., 2006. Reduction of crying episodes owing to infantile colic: A randomized controlled study on the efficacy of a new infant formula. Eur J Clin Nutr 60, 1304–1310. https://doi.org/10.1038/SJ.EJCN.1602457

Sheng, X.Y., Buthmanaban, V., Van Lieshout, G.A.A., Parikh, P., 2020. Reduced Occurrence of Gastrointestinal Symptoms in Chinese Infants Fed Minimally Processed Commercially Available Formula: A Cross-Sectional Observational Study. J Nutr Metab 2020. https://doi.org/10.1155/2020/1807397

Slavin, J., 2013. Fiber and prebiotics: mechanisms and health benefits. Nutrients 5, 1417–1435. https://doi.org/10.3390/NU5041417

Sun, W., Wang, Z., Zhong, M., Xue, Q., Chen, Y., Deng, M., Chen, H., He, P., Wen, X., Tan, M.-Y., Liu, Q., Li, Q., Liu, Y., Xu, C., Xu, H.-Q., Liu, Z., Wang, W., Yan, Y., Lin, R.-B., He, J., Li, P., 2015. Preventive effect of prenatal education and partially hydrolyzed whey formula on infantile allergy: a prospective study. Chinese Journal of Perinatal Medicine. https://doi.org/10.3760/CMA.J.ISSN.1007-9408.2015.12.005

Szajewska, H., Horvath, A., 2010. Meta-analysis of the evidence for a partially hydrolyzed 100% whey formula for the prevention of allergic diseases. Curr Med Res Opin 26, 423–437. https://doi.org/10.1185/03007990903510317

Taylor, S.L., 1986. Immunologic and Allergic Properties of Cows' Milk Proteins in Humans. J Food Prot 49, 239–250. https://doi.org/10.4315/0362-028X-49.3.239

van Lieshout, G.A.A., Lambers, T.T., Bragt, M.C.E., Hettinga, K.A., 2020. How processing may affect milk protein digestion and overall physiological outcomes: A systematic review. Crit Rev Food Sci Nutr 60, 2422–2445. https://doi.org/10.1080/10408398.2019.1646703

Van Tilburg, M.A.L., Rouster, A., Silver, D., Pellegrini, G., Gao, J., Hyman, P.E., 2016. Development and validation of a Rome III functional gastrointestinal disorders questionnaire for infants and toddlers. J Pediatr Gastroenterol Nutr 62, 384–386. https://doi.org/10.1097/MPG.0000000000000962

Vandenplas, Yvan, Bhatia, J., Shamir, R., Agostoni, C., Turck, D., Staiano, A., Szajewska, H., 2014. Hydrolyzed formulas for allergy prevention. J Pediatr Gastroenterol Nutr 58, 549–552. https://doi.org/10.1097/MPG.0000000000000318

Vandenplas, Y., Cruchet, S., Faure, C., Lee, H.C., Di Lorenzo, C., Staiano, A., Chundi, X., Aw, M.M., Gutiérrez-Castrellõn, P., Asery, A., Spolidoro, J., Heine, R.G., Miqdady, M., Arancibia, M.E., Alarcõn, P., 2014. When should we use partially hydrolysed formulae for frequent

gastrointestinal symptoms and allergy prevention? Acta Paediatr 103, 689–695. https://doi.org/10.1111/APA.12637

Vandenplas, Y., Munasir, Z., Hegar, B., Kumarawati, D., Suryawan, A., Kadim, M., Djais, J.T., Basrowi, R.W., Krisnamurti, D., 2019. A perspective on partially hydrolyzed protein infant formula in nonexclusively breastfed infants. Korean J Pediatr. https://doi.org/10.3345/kjp.2018.07276

Victora, C.G., Bahl, R., Barros, A.J.D., França, G.V.A., Horton, S., Krasevec, J., Murch, S., Sankar, M.J., Walker, N., Rollins, N.C., Allen, K., Dharmage, S., Lodge, C., Peres, K.G., Bhandari, N., Chowdhury, R., Sinha, B., Taneja, S., Giugliani, E., Horta, B., Maia, F., de Mola, C.L., Hajeebhoy, N., Lutter, C., Piwoz, E., Martines, J.C., Richter, L., 2016. Breastfeeding in the 21st century: epidemiology, mechanisms, and lifelong effect. Lancet 387, 475–490. https://doi.org/10.1016/S0140-6736(15)01024-7

Von Berg, A., Koletzko, S., Grübl, A., Filipiak-Pittroff, B., Wichmann, H.E., Bauer, C.P., Reinhardt, D., Berdel, D., 2003. The effect of hydrolyzed cow's milk formula for allergy prevention in the first year of life: The German Infant Nutritional Intervention Study, a randomized double-blind trial. Journal of Allergy and Clinical Immunology 111, 533–540. https://doi.org/10.1067/mai.2003.101

Weaver, L.T., Ewing, G., Taylor, L.C., 1988. The bowel habit of milk-fed infants. J Pediatr Gastroenterol Nutr 7, 568–571. https://doi.org/10.1097/00005176-198807000-00015

WHO, 2010. WHO Anthro for personal computers, version 3.2.2, 2011: Software for assessing growth and development of the world's children. Geneva [WWW Document]. URL http://www.who.int/childgrowth/software/en/ (accessed 4.4.23).

WHO, 2009. WHO Child Growth Standards. Dev Med Child Neurol 51, 1002–1002. https://doi.org/10.1111/J.1469-8749.2009.03503.X

WHO Multicentre Growth Reference Study Group, 2006. WHO Child Growth Standards - Length/Height-for-age, Weight-for-age, Weight-for-length, Weight-for-height and Body Mass Index-for age : Methods and Development. [WWW Document]. URL https://www.who.int/publications/i/item/924154693X (accessed 3.30.23).

Wu, S.L., Ding, D., Fang, A.P., Chen, P.Y., Chen, S., Jing, L.P., Chen, Y.M., Zhu, H.L., 2017. Growth, gastrointestinal tolerance and stool characteristics of healthy term infants fed an infant formula containing hydrolyzed whey protein (63%) and intact casein (37%): A randomized clinical trial. Nutrients 9. https://doi.org/10.3390/nu9111254

Yao, M., Lien, E.L., Capeding, M.R.Z., Fitzgerald, M., Ramanujam, K., Yuhas, R., Northington, R., Lebumfacil, J., Wang, L., DeRusso, P.A., 2014. Effects of term infant formulas containing high sn-2 palmitate with and without oligofructose on stool composition, stool characteristics, and bifidogenicity. J Pediatr Gastroenterol Nutr 59, 440–448. https://doi.org/10.1097/MPG.0000000000000443

APPENDIX A

Published manuscripts.

- **Manuscript 1**

Manios *et al. BMC Nutrition* (2020) 6:46
https://doi.org/10.1186/s40795-020-00365-4

BMC Nutrition

RESEARCH ARTICLE Open Access

Effect of milk fat-based infant formulae on stool fatty acid soaps and calcium excretion in healthy term infants: two double-blind randomised cross-over trials

Yannis Manios[1], Eva Karaglani[1], Inge Thijs-Verhoeven[2], Elpis Vlachopapadopoulou[3], Anastasia Papazoglou[1], Eleni Maragoudaki[1], Zafeiris Manikas[1], Tarek-Michail Kampani[1], Iliana Christaki[1], Marlotte M. Vonk[2], Rolf Bos[2] and Panam Parikh[2*]

Abstract

Background: Palmitic acid (PA) is predominantly esterified at the SN-2 position of triacylglycerols in human milk. PA at the SN-2 position is more efficiently absorbed and results in reduced formation of PA soaps, as well as reduced fatty acid (FA) and calcium malabsorption. Bovine milk fat (MF), a natural source of SN-2-palmitate, was used in the fat blend of infant formulae (IF) in the current study to investigate its effect on stool fatty acid soaps, calcium excretion and stool characteristics.

Methods: Two double-blind, randomised cross-over trials (CS1, CS2) were conducted in parallel with healthy term, formula-fed infants aged 9–14 weeks. After a two-week run-in period, infants in CS1 ($n = 17$) were randomly allocated to receive either a 50% MF-based formula (50MF) or a 100% vegetable fat (VF) formula; in CS2 ($n = 18$), infants received either a 20% MF-based formula (20MF) or the VF formula, in a 2 × 2-week cross-over design. At the end of each two-week intervention period, stool samples were collected for FA, FA soaps and calcium excretion analysis and stool consistency was assessed according to the Amsterdam Infant Stool Scale (AISS).

Results: MF-based groups showed no significant difference in PA in stools compared to VF group, although reduced stool PA soaps (CS1: 111.28 ± 18.33 vs. 220.25 ± 29.35 mg/g dry weight, $p < 0.0001$; CS2: 216.24 ± 25.16 vs. 233.94 ± 35.12 mg/g dry weight, $p = 0.0023$), total FA soaps and calcium excretion (CS1: 46.40 ± 5.27 vs. 49.88 ± 4.77 mg/g dry weight, $p = 0.0041$; CS2: 46.20 ± 4.26 vs. 50.47 ± 6.71 mg/g dry weight, $p = 0.0067$) were observed. Furthermore, the 50MF group showed a favourable lower mean stool consistency score compared to the VF group (1.64 ± 0.49 vs. 2.03 ± 0.19, $p = 0.0008$).

Conclusions: While the use of bovine MF in IF did not affect PA concentrations in stool, lower excretion of palmitate soaps, total FA soaps and calcium was seen in healthy term infants. 50MF formula also showed improved stool consistency. The use of MF in IF could be an interesting approach to improve gut comfort and stool characteristics in infants, warranting further research.

(Continued on next page)

* Correspondence: panam.parikh@frieslandcampina.com
[2]FrieslandCampina, Stationsplein 1, 3818 LE Amersfoort, the Netherlands
Full list of author information is available at the end of the article

(Continued from previous page)
Trial registration: Netherlands Trial Registry Identifier: NTR6702. Date registered: December 01, 2017.

Keywords: Milk fat, SN-2-palmitate, Palmitate soap, Calcium excretion, Stool consistency, Amsterdam infant stool scale

Background

Human milk (HM) represents optimum nutrition for full-term babies throughout infancy and is designed to meet the needs of the growing infant in the first months after birth [1]. Triacylglycerols (TAGs) in HM provide approximately 50% of the energy as well as essential fatty acids (FAs) important for the overall development of the infant [2–4]. Palmitic acid (PA), one of the major saturated fatty acids in HM (representing approximately 20–25% of total FAs), is predominantly esterified at the SN-2 position of TAGs (i.e. SN-2-palmitate) in HM. [1, 2, 5] Studies over the last two to three decades have provided increasing evidence that the SN-2-predominant positioning of PA in HM TAGs promotes the absorption of both PA and calcium in term and preterm infants [3, 6–8].

The majority of infant formulae (IF) use a blend of vegetable oils as a source of fat. Compared to HM fat, in which 70–88% of the PA is esterified at the SN-2 position, commonly used vegetable oils have lower percentage of PA in the SN-2 position of TAGs (10–20%) [5]. Therefore, vegetable fat (VF) blends consist of TAGs with PA predominantly bound to the SN-1 and SN-3 positions [5, 9]. During digestion, PA at the SN-1,3 positions is released as free PA. In the alkaline environment of the small intestinal lumen, free PA interacts readily with cations (e.g. calcium) to form insoluble soaps [10, 11] that are associated with hard stools, gut discomfort and decreased absorption of PA and minerals by the infant [8, 11, 12]. Increasing the ratio of SN-2 to SN-1 and SN-3 palmitate in IF could ensure higher absorption of fat and minerals (calcium), as well as lead to reduced formation of insoluble soaps, thereby, minimizing gut discomfort.

Synthetic structured TAGs have been developed with higher proportion of PA in the SN-2 position (ranging from 35.9–74%) and lower levels of PA at the SN-1 and SN-3 positions. Favourable effects of IF containing such synthetic TAGs on FA, calcium absorption and stool consistency have been reported in healthy infants by several studies [6, 7, 13–19].

Bovine milk fat (MF) is naturally higher in SN-2-palmitate than VFs, with a level of approximately 40% [8, 9, 11] and a higher ratio of SN-2 vs SN-1,3 palmitate. Furthermore, MF shows comparable TAG structures to those in HM fat [8]. Therefore, using MF in the development of IF may enable mimicking the composition and structure of HM fat, potentially leading to a higher absorption of PA and calcium, less soap formation and softer stools in comparison to IF containing VF only.

This paper reports on two studies. Each study was a double-blind, cross-over, randomised, placebo-controlled comparing a MF-based formula against a standard VF formula. The primary objective of these studies was to evaluate the excretion of PA and PA soaps in stools of healthy term infants. We hypothesised that infants fed MF-based IF had lower PA and PA soaps in stool when compared to infants fed VF-based formula. In addition, the secondary outcomes of both studies were calcium excretion in stools, stool consistency scores and other FA and FA soaps in stools.

Methods

Study design and population

The present studies were two separate double-blind, cross-over, randomised, placebo-controlled trials, conducted in parallel with healthy, full-term, exclusively formula-fed (FF) infants (Fig. 1). Sampling and recruitment were performed by paediatricians at 12 private paediatric clinics in two cities (Athens and Larissa) in Greece between December 2017 and July 2018. Infants were screened between their 9th–14th week of age on the following inclusion criteria: full-term, healthy (born at gestational age ≥ 37 weeks), exclusively FF infants, with appropriate for gestational age birthweight. Exclusion criteria were: i) severe acquired or congenital diseases, mental or physical disorders, any symptoms of allergy (including cow's milk allergy); ii) Use of probiotics, antibiotics or other medication that treat or cause GI symptoms; iii) use of medication(s) known or suspected to affect fat digestion, absorption and/or metabolism, nutritional supplements, suppositories, medication that may suppress or neutralize gastric acid secretion and gut motility at the time of screening or at any time throughout the study period; iv) participation in another clinical trial; v) any type of mixed feeding (See eMethods 1 for full inclusion and exclusion criteria). Written informed consent was obtained from parents after explanation of the study procedures and prior to inclusion into the study. The study procedures were initiated immediately upon inclusion. The protocol, information letter to the parents/caregivers and written informed consent form were approved by Harokopio University's Ethics Committee. The study was conducted in accordance with the guidelines of the Declaration of Helsinki and the International Conference

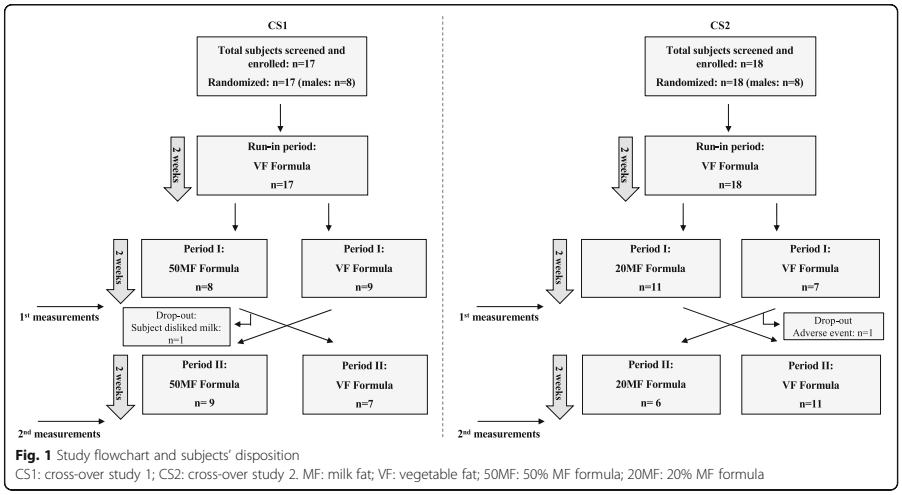

Fig. 1 Study flowchart and subjects' disposition
CS1: cross-over study 1; CS2: cross-over study 2. MF: milk fat; VF: vegetable fat; 50MF: 50% MF formula; 20MF: 20% MF formula

on Harmonisation (ICH) guidelines on Good Clinical Practice (GCP) and was registered in the Netherlands Trial Registry (identifier: NTR6702).

Study randomisation and formulae

Upon inclusion in the study, all infants were fed the 100% VF formula with 10.1% SN-2-palmitate levels (; total PA 24.9%) for 2 weeks (run-in period) in order to minimize the potential effects of previous feedings. Infants were then allocated to one of the cross-over studies using block randomisation. In each of the studies infants were randomly assigned to receive either the VF formula or a MF-based formula: i) 50% MF + 50% VF (50MF) with 39% SN-2-palmitate levels (total PA 18.9%) in cross-over study 1 (CS1) and ii) 20% MF + 80% VF (20MF) with 19.7% SN-2-palmitate levels (total PA 26.1%) in cross-over study 2 (CS2). Randomisation into the two treatment arms per study was based on a computer-generated sequence. After 2 weeks (period I), infants were crossed over to receive the other formula for another 2 weeks (period II) in their respective CS1 and CS2 (Fig. 1). The nutritional composition of the three study formulae was similar with the only difference being their FA profiles and percentage of SN-2-palmitate (Table 1). The procedures followed for the determination of SN-2-palmitate and total FA profile of study products can be found in eMethods 2. All powder properties were identical between the control and experimental formulae. All formulae were produced in the Netherlands by FrieslandCampina and were packaged in similar blank tins of 400 g each with a specific identification code at the bottom of the tins. The study formulae were labelled by the manufacturer using a single letter per formula group (A, B, C, D or E). The manufacturer retained the codes for the study formulae. All study personnel, including the Principal Investigator and the Sponsor's Project Manager as well as parents/caregivers were blinded to the formulae allocation. Sealed envelopes containing product codes were provided to the study site in the event of an emergency. The tin label included guidance for the parents on the daily volume of formula intake required by the infant, which depended upon age and weight.

Stool collection and analysis

Stool samples were collected at home by parents/caregivers for three consecutive days at the end of period I and period II for analysis of their FAs, FA soaps and calcium content. Each freshly passed stool was placed in a faecal tube collector (until 30 g was collected in total), kept in a ziplock amber plastic bag and then stored in the home freezer. At the end of each intervention period, the study personnel collected the stool samples from the homes and brought them to Harokopio University. The stool samples were stored in Harokopio University in a freezer at -80 °C until being transported in dry ice to Covance Laboratory, Madison, Wisconsin, USA for analysis. The analytical procedures followed in the laboratory are described in eMethods 2.

Table 1 Composition of the study formulae

	Formula		
Nutrient/ingredient	50MF	20MF	VF
Energy (kcal/100 mL)	66	66	66
Intact protein (g/100 mL)	1.4	1.4	1.4
Carbohydrates (g/100 mL)	7.1	7.0	7.0
Galacto-oligosaccharides (g/100 mL)	0.27	0.27	0.27
Fat (g/100 mL)	3.5	3.5	3.5
Docosahexanoic acid (mg/100 mL)	6.9	6.9	6.9
Arachidonic acid (mg/100 mL)	8.3	8.3	6.9
Fatty acids; mol % of TAGs			
C12:0; Lauric acid	6.0	7.7	10.4
C14:0; Myristic acid	7.4	4.8	3.9
C16:0; Palmitic acid	18.9	26.1	24.9
C18:0; Stearic acid	5.2	4.4	3.4
C18:1; Oleic acid	36.9	42.2	39.0
C18:2; Linoleic acid	11.7	16.4	12.7
C18:3; a-Linolenic acid	1.5	1.6	1.8
C20:0; Arachidic acid	0.2	0.3	0.3
% C16:0 in sn-2 position	39	19.7	10.1
Calcium (mg/100 mL)	53	55	56

MF milk fat; *VF* vegetable fat. *50MF* 50% MF formula; *20MF* 20% MF formula
To ensure double-blindness, all formulae were packaged in similar blank tins of 400 g each with different identification codes at the bottom of the tins. Formula labels provided preparation, storage and feeding instructions in English and Greek

Formula consumption and stool characteristics

Parents/caregivers were asked to record formula consumption using a three-day milk diary, where the timing, frequency as well as the exact amount/volume (in mL) of formula consumed were recorded during the same 3 days of each intervention period as stool collection. Additionally, the study personnel collected all formula tins to monitor compliance and formula consumption.

Stool characteristics assessment was performed by parents/caregivers using the validated Amsterdam Infant Stool Scale (AISS) [20], which assesses the consistency, amount/volume and colour of stools. For assessment of consistency, each freshly passed stool during the three-day period was evaluated and ranked accordingly on a scale of one to four (watery = 1, soft = 2, formed = 3, hard =4) and a mean score was calculated.

Safety and anthropometric assessment

Adverse events (AEs) and serious adverse events (SAEs) were recorded throughout the study and monitored by an independent paediatrician. No code-break requests occurred for AEs or SAEs throughout the study and de-blinding did not need to take place. Anthropometric indices (weight and length) were also measured following standardized procedures at screening and at the end of the run-in period, period I and period II.

Statistical analysis

Sample size for both studies was determined based on the data from one available cross-over study by Carnielli et al. 1995 [14] on the concentration of PA in stools in infants fed control and high SN-2-palmitate formula, and adjusted for dose and duration. At least 16 infants per cross-over study were required to achieve a power of 80% ($\alpha = 0.05$) to detect a mean (SD) between-group difference of 25 (13.9) mg PA per /g of wet stool between VF control IF and MF-based IF. Assuming an expected 30% drop-out rate, 22 infants per cross-over study were required to achieve 16 evaluable infants per cross-over study. Data analyses were performed with the study groups coded; the code was not broken until all analyses had been completed.

The two cross-over studies were analysed independently from each other by 4Pharma Ltd. (Finland) using SAS® version 9.4 for Windows (SAS Institute Inc., Cary, NC, USA). The primary outcomes were excretion of PA and PA soaps in stool. A hierarchical approach was taken when interpreting the results, with PA in stool tested first for statistical significance, followed by PA soaps in stool. Therefore, no further adjustments for multiplicity were conducted on the *p*-values. ANOVA appropriate for a 2 × 2 cross-over design was used to assess mean differences in stool PA and PA soap composition. When the normality assumption was not met, variables were log-transformed or Wilcoxon signed-rank test was applied. The statistical model included treatment, sequence and period as fixed effects, and subject (sequence) and residual error term as random effects.

The secondary outcomes were calcium absorption and stool consistency (using AISS). The same ANOVA approach was used for calcium excretion and stool consistency analysis. Milk intake comparisons between the formula groups was done using Mann-Whitney U-test. All statistical tests were two-sided and performed with $\alpha = 0.05$.

Additional exploratory analyses were performed on total FA, total FA soaps, FA and FA soaps (ANOVA as with primary outcomes).

Results

Study population

From the total infants enrolled in CS1 and CS2 ($n = 17$ and $n = 18$, respectively), one infant dropped out of CS1 (subject disliked milk) and one from CS2 (subject had adverse event, not related to study product). The total number of infants that completed CS1 and CS2 was $n = 16$ and $n = 17$, respectively (Fig. 1). It was decided to

stop recruitment when each cross-over study had at least 16 infants completing the study. The overall drop-out rate was below 10% (2 subjects dropped out).

The baseline and family characteristics of the subjects are descriptively presented in Table 2. Weight at birth, gestational age as well as infants age and weight at inclusion were similar among the groups per cross-over study.

Formula consumption and anthropometric data

The average weekly milk intake or the subjects' weight and length measurements at the end of the two-week intervention periods did not differ between the MF and VF groups in either of the cross-over studies (eTable 3).

Stool fatty acids

The faecal concentrations of the major FAs are reported in Table 3. No significant difference was noted in the PA in stool between the MF-based IF and VF formula in both, CS1 and CS2. Similarly, no difference was observed for the total free FAs between the MF-based IF and VF formula.

The MF-based IF group in both cross-over studies had lower Lauric acid (C12:0) concentrations (CS1: $p < 0.0001$; CS2: $p = 0.004$) than VF group. In contrast, the opposite was observed for Myristic (C14:0) and Stearic (C18:0) in the MF-based IF groups ($p < 0.05$) in both, CS1 and CS2. The 50MF group (CS1) also had higher level of Gamma Linolenic acid than the VF group ($p < 0.05$).

In addition, Table 3 presents the faecal concentrations of the major FAs as the % of each FA within total free FAs lost in one g of dry stool. In CS1, the 50MF group had a decreased % of PA ($p = 0.0003$) and Lauric acid ($p < 0.0001$), and increased % of Myristic and Stearic acids ($p < 0.0001$) compared to the VF group. In CS2, no differences were observed in the % of PA, however, a decreased % of Lauric acid was observed in the 20MF group compared to the VF group ($p = 0.0002$).

Stool fatty acid soaps

The MF-based IF groups in both CS1 and CS2 had a lower concentration of total FA soaps in stool than the VF group (Table 3; CS1: $p < 0.0001$; CS2: $p = 0.0077$). In CS1, the 50MF group had a lower concentration of PA soaps in stool compared to the VF group ($p < 0.0001$). Similar results were also noted in CS2, with lower PA soaps in the 20MF group ($p = 0.0023$). In CS1, Lauric acid (C12:0) soap concentrations were lower ($p < 0.0001$), whilst Stearic acid (C18:0) soap concentration was increased in the 50MF group compared to the VF group ($p < 0.0001$). In CS2, a decrease in Lauric (C12:0), Oleic (C18:1) and Linoleic acid (C18:2) soap concentrations were observed in the 20MF group compared to the VF group ($p < 0.05$). Stearic acid (C18:0) soap concentration, however, was increased ($p = 0.0021$) (Table 3).

In addition, Table 4 presents the faecal concentrations of the major FA soaps as the % of each FA soap within total FA soaps lost in one g of dry stool. In CS1 and CS2 both, 50MF and 20MF groups had decreased % of PA soaps compared to the VF group (CS1: $p < 0.0001$; CS2: $p = 0.0032$). In CS1, similar results were observed for the % of Lauric acid (C12:0) soaps ($p < 0.0001$), while the opposite was observed for Myristic (C14:0), Stearic (C18:0) and Oleic acid (C18:1) soaps ($p < 0.0001$). In CS2, a decrease was observed for the % of Lauric (C12:0) and Linoleic acid (C18:2) soaps ($p < 0.0001$ and $p = 0.0059$,

Table 2 Baseline infant and family characteristics

	CS1		CS2	
	50MF - VF ($n = 7$)	VF – 50MF ($n = 9$)	20MF - VF ($n = 11$)	VF - 20MF ($n = 6$)
Gender, No. (%) male	3 (43)	5 (56)	6 (55)	2 (33)
Age at screening, mean (SD), days	103 (16)	92 (22)	95 (18)	96 (17)
Weight at screening, mean (SD), g	6368 (798)	5380 (1018)	5941 (1105)	5192 (722)
Mother's age, mean (SD), years	34 (7)	32 (5)	35 (8)	33 (4)
Mother's education level:				
No. (%) < 12 years	2 (29)	3 (33)	5 (46)	1 (17)
No. (%) 12–14 years	2 (29)	2 (22)	1 (9)	3 (50)
No. (%) > 14 years	3 (43)	4 (44)	5 (46)	2 (33)
Gestational age, mean (SD), weeks	39 (2)	38 (1)	39 (1)	38 (1)
Mode of delivery				
No. (%) caesarean section	4 (57)	7 (78)	6 (55)	5 (83)
Weight at birth, mean (SD), g	3259 (491)	2883 (391)	3143 (399)	2833 (318)

Data are descriptively summarized, given the cross-over design of the study
CS1 cross-over study 1; *CS2* cross-over study 2; *SD* standard deviation; *50MF* 50% MF formula; *20MF* 20% MF formula; *MF* milk fat; *VF* vegetable fat

Table 3 Stool fatty acids, fatty acid soaps and calcium composition (mg/g stool dry weight)

CS1			CS2		
	50MF (*N* = 16)	VF (*N* = 16)		20MF (*N* = 17)	VF (*N* = 17)
Free Fatty Acids			Free Fatty Acids		
Palmitic acid (C16:0)[2]	4.4 (3.4–10.3)	5.7 (4.4–9.1)	Palmitic acid (C16:0)[3]	5.9 (3.8–13.4)	4.9 (3.8–7.3)
Lauric acid (C12:0)[2]	0.50 (0.28–0.78)[1]	1.38 (1.11–1.99)	Lauric acid (C12:0)[1]	1.30 (0.72)[2]	1.59 (0.840
Myristic acid (C14:0)[1]	1.35 (0.70)[2]	1.00 (0.59)	Myristic acid (C14:0)[3]	0.98 (0.66–1.59)[2]	0.79 (0.64–1.00)
Stearic acid (C18:0)[2]	1.83 (1.25–4.37)[2]	1.25 (0.93–1.84)	Stearic acid (C18:0)[3]	1.40 (0.92–2.94)[2]	0.99 (0.83–1.48)
Oleic acid (C18:1 n-9)[2]	4.80 (3.32–7.84)	5.01 (3.91–8.30)	Oleic acid (C18:1 n-9)[3]	6.65 (4.09–8.29)	5.70 (4.65–7.43)
Linoleic acid (C18:2)[2]	0.73 (0.46–1.36)	0.84 (0.45–1.46)	Linoleic acid (C18:2)[2]	0.93 (0.72–1.95)	0.88 (0.84–1.37)
Gamma Linolenic acid (C18:3 n-6)[1]	0.08 (0.02)[2]	0.07 (0.02)	Gamma Linolenic acid (C18:3 n-6)[1]	0.09 (0.04)	0.08 (0.02)
Alpha Linolenic acid (C18:3 n-3)[3]	0.07 (0.07–0.10)	0.07 (0.06–0.11)	Alpha Linolenic acid (C18:3 n-3)[2]	0.09 (0.07–0.19)	0.09 (0.08–0.15)
Arachidic acid (C20:0)[2]	0.10 (0.07–0.18)	0.10 (0.09–0.17)	Arachidic acid (C20:0)[3]	0.09 (0.07–0.17)	0.09 (0.08–0.12)
Total FAs[1]	22.37 (11.43)	23.16 (12.84)	Total FAs[3]	18.6 (15.7–32.7)	19.4 (15.3–22.3)
Fatty Acid Soaps			Fatty Acid Soaps		
Palmitic soap (C16:0)[1]	111.28 (18.33)[1]	220.25 (29.35)	Palmitic soap (C16:0)[1]	216.24 (25.16)[2]	233.94 (35.12)
Lauric soap (C12:0)[2]	1.76 (1.50–2.27)[1]	6.83 (5.74–7.67)	Lauric soap (C12:0)[1]	4.38 (1.27)[1]	7.34 (1.88)
Myristic soap (C14:0)[1]	10.82 (2.09)	11.24 (1.37)	Myristic soap (C14:0)[3]	11.90 (10.90–13.20)	12.20 (11.10–12.70)
Stearic soap (C18:0)[1]	50.92 (7.81)[1]	31.21 (4.78)	Stearic soap (C18:0)[3]	39.50 (38.40–46.40)[2]	36.40 (31.20–37.60)
Oleic soap (C18:1 n-9)[2]	10.02 (7.05–14.05)	8.72 (7.61–12.65)	Oleic soap (C18:1 n-9)[1]	10.10 (6.11)[2]	11.63 (7.29)
Linoleic soap (C18:2)[2]	1.11 (0.70–1.42)	1.13 (0.92–1.47)	Linoleic soap (C18:2)[1]	1.21 (0.70)[2]	1.57 (0.98)
Total FA soaps[1]	201.63 (34.79)[1]	290.19 (42.81)	Total FA soaps[1]	296.59 (31.29)[2]	311.18 (39.75)
Calcium			Calcium		
Stool calcium[1]	46.40 (5.27)[2]	49.88 (4.77)	Stool calcium[1]	46.20 (4.26)[2]	50.47 (6.71)

[1]Analysis of variance for variable in original scale of measurement. Data are presented as mean (SD)
[2] Analysis of variance for log-transformed variable. Data are presented as median (IQR)
[3]Non-parametric analysis (Wilcoxon Signed Rank). Data are presented as median (IQR)
P-values indicated by a, $p < 0.0001$; b, $p < 0.05$ are not eligible for statistical significance according to pre-defined hierarchy
CS1 cross-over study 1; *CS2* cross-over study 2; *50MF* 50% MF formula; *20MF* 20% MF formula; *MF* milk fat; *VF* vegetable fat; *SD* standard deviation; *IQR* inter-quartile range

respectively), while the opposite was observed for Myristic (C14:0) and Stearic acid (C18:0) soaps ($p = 0.0058$ and $p = 0.0026$, respectively).

Stool calcium

The mean calcium concentration in stools was lower in both 50MF and 20MF groups compared to their respective VF group (CS1: $p = 0.0041$; CS2: $p = 0.0067$; Table 3).

Stool consistency

The mean stool consistency is presented in Fig. 2. In CS1, the mean stool consistency score was decreased in 50MF group compared to the VF group ($p = 0.0032$). Parents/caregivers of infants in the 50MF group reported watery and soft stools, while the VF group reported only soft stools. The mean stool consistency score in CS2 did not differ between the 20MF and VF groups, and was classified as soft.

Discussion

To our knowledge, this is the first study assessing the effect of IF with bovine MF on stool FAs, FA soaps and calcium excretion in healthy term infants. Although, current studies did not show a significant difference on PA in stool as initial primary outcome measure, an interesting observation is that both, 50MF and 20MF formulae did demonstrate favourable effects on PA soaps in stool and other secondary outcomes, e.g. calcium excretion and total FA soaps in stools, compared to the VF formula. This underlines the importance of further exploration of bovine MF application in IF. Additionally, various FA showed different trends in FA soap concentrations with increase of MF content in the IF. As the IF in the current study differed in their overall FA profile, it is likely that this contributed to the observed FA trends and not just their distribution over SN-2 and SN-1,3 positions.

Table 4 Percentages of individual FAs and FA soaps within total free FAs and total FA soaps, respectively

CS1			CS2		
	50MF (*N* = 16)	VF (*N* = 16)		20MF (*N* = 17)	VF (*N* = 17)
% Individual Fatty Acids within Total Free FAs			% Individual Fatty Acids within Total Free FAs		
% Palmitic acid (C16:0)[1]	28.79 (8.41)[2]	35.88 (10.46)	% Palmitic acid (C16:0)[3]	31.2 (23.0–36.0)	29.3 (24.3–36.0)
% Lauric acid (C12:0)[1]	2.39 (0.73)[1]	7.05 (1.94)	% Lauric acid (C12:0)[1]	4.99 (1.78)[2]	7.28 (2.25)
% Myristic acid (C14:0)[1]	6.06 (1.01)[1]	4.26 (0.56)	% Myristic acid (C14:0)[1]	4.44 (0.92)	4.08 (0.62)
% Stearic acid (C18:0)[1]	11.57 (3.96)[1]	7.20 (1.94)	% Stearic acid (C18:0)[3]	7.43 (5.64–8.23)	5.73 (5.45–6.76)
% Oleic acid (C18:1 n-9)[1]	29.74 (10.25)	28.23 (9.07)	% Oleic acid (C18:1 n-9)[1]	31.11 (7.95)	28.51 (7.71)
% Linoleic acid (C18:2)[1]	4.66 (2.63)	4.39 (1.84)	% Linoleic acid (C18:2)[1]	5.68 (2.47)	5.79 (2.54)
% Gamma Linolenic acid (C18:3 n-6)[2]	0.37 (0.30–0.57)	0.35 (0.27–0.41)	% Gamma Linolenic acid (C18:3 n-6)[2]	0.32 (0.26–0.47)	0.35 (0.32–0.47)
% Alpha Linolenic acid (C18:3 n-3)[1]	0.50 (0.215)	0.44 (0.18)	% Alpha Linolenic acid (C18:3 n-3)[1]	0.52 (0.23)	0.57 (0.27)
% Arachidic acid (C20:0)[1]	0.58 (0.15)	0.59 (0.19)	% Arachidic acid (C20:0)[2]	0.52 (0.46–0.60)	0.54 (0.46–0.64)
% Fatty Acid Soaps within Total FA Soaps			% Fatty Acid Soaps within Total FA Soaps		
% Palmitic soap (C16:0)[3]	54.4 (54.1–57.3)[1]	76.2 (75.6–77.6)	% Palmitic soap (C16:0)[3]	72.7 (71.5–74.7)[2]	76.6 (74.0–77.3)
% Lauric soap (C12:0)[1]	0.93 (0.25)[1]	2.46 (0.38)	% Lauric soap (C12:0)[1]	1.48 (0.42)[1]	2.36 (0.53)
% Myristic soap (C14:0)[1]	5.36 (0.35)[1]	3.89 (0.15)	% Myristic soap (C14:0)[3]	4.05 (3.88–4.12)[2]	3.93 (3.71–3.96)
% Stearic soap (C18:0)[2]	25.52 (23.95–26.48)[1]	10.73 (10.34–11.01)	% Stearic soap (C18:0)[3]	14.04 (13.10–14.87)[2]	11.24 (10.31–11.78)
% Oleic soap (C18:1 n-9)[1]	5.45 (2.17)[2]	3.94 (1.88)	% Oleic soap (C18:1 n-9)[1]	3.38 (1.77)	3.72 (2.14)
% Linoleic soap (C18:2)[1]	0.62 (0.29)	0.50 (0.26)	% Linoleic soap (C18:2)[1]	0.40 (0.21)[2]	0.51 (0.29)

[1] Analysis of variance for variable in original scale of measurement. Data are presented as mean (SD)
[2] Analysis of variance for log-transformed variable. Data are presented as median (IQR)
[3] Non-parametric analysis (Wilcoxon Signed Rank). Data are presented as median (IQR)
P-values indicated by a, $p < 0.0001$; b, $p < 0.05$ are not eligible for statistical significance according to pre-defined hierarchy.
CS1 cross-over study 1; *CS2* cross-over study 2; *50MF* 50% MF formula; *20MF* 20% MF formula; *MF* milk fat; *VF* vegetable fat; *SD* standard deviation; *IQR* inter-quartile range

Interestingly, 50MF formula with high SN-2-palmitate levels favourably affected infants' stool consistency scores. These findings are in line with published literature, although the reported studies had different study designs, age groups of infants and/or duration of interventions [6, 7, 14–16, 18, 19]. Most of these studies have tested IF with synthetic TAGs at various proportions of SN-2-palmitate, in contrast to the current MF-based formulae.

All previous studies consistently report that a higher SN-2-palmitate content in IF results in improved PA and FAs absorption [14, 15, 18] or lower faecal excretion, either as free PA and free FAs [6, 14] or as PA soaps and FA soaps in the faeces [6, 7, 16, 19]. No differences were observed between the current test groups and their respective control group on the absolute PA concentrations in the faeces, only the proportion of PA within total FAs excreted in the faeces was lower in the 50MF group compared to the VF group. However, infants fed with both MF-based formulae, despite lower SN-2-palmitate levels than reported in literature for synthetic TAGs [6, 7, 14–16, 18, 19], had lower amounts of PA soaps in their stools compared to the VF formula. Furthermore, infants fed 20MF also had lower faecal excretion of Oleic and Linoleic soaps compared to those receiving VF formula which can be speculated as an additional benefit of the increased SN-2-palmitate content using MF on the absorption of these essential FAs. This suggests that increasing the SN-2-palmitate content through the use of MF might have comparable favourable effects to synthetic TAGs even at a lower concentration.

Calcium excreted in the faeces was found to be lower in both MF groups compared to the VF group. This potentially suggests improved calcium absorption by the infants as reported by previous balance studies [14, 15, 18]. This finding is particularly relevant since the groups had comparable average IF intake and the calcium content in the formulae was similar. The potential health benefits of improved calcium availability on bone indices

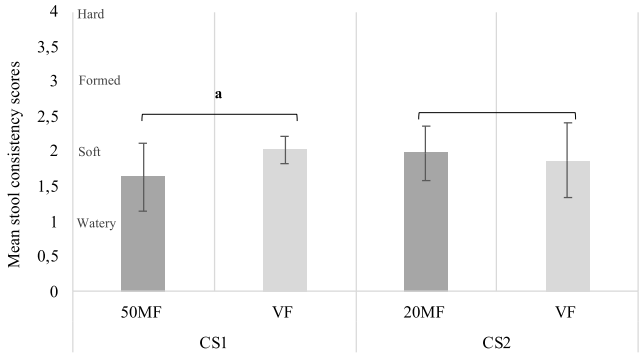

Fig. 2 Stool consistency scores according to feeding group
Individual stool consistency scores were determined using the Amsterdam Infant Stool Scale (AISS) (categorization: 1 = watery, 2 = soft, 3 = formed, and 4 = hard). Comparisons between the formula groups were conducted using analysis of variance. Values are mean (SD)
CS1: cross-over study 1; CS2: cross-over study 2. MF: milk fat; VF: vegetable fat; SD standard deviation. Significant difference between the 50MF and the VF group: $^{a}p = 0.0032$; 50MF: 50% MF formula; 20MF: 20% MF formula

have been reported by two previous studies in healthy term infants which showed improved bone mass / bone strength / quality (as determined either by dual-energy x-ray absorptiometry [16] or by quantitative ultrasound measurements of bone speed of sound [21]) when a high (50 and 43%, respectively) SN-2-palmitate formula was used compared to a standard low (12 and 14%, respectively) SN-2-palmitate formula. A balance study to confirm whether the reduced faecal calcium excretion seen in this study correlates with improved calcium retention and absorption is warranted.

In this study we have used the AISS [20], which is considered a more appropriate tool for infants defecating in nappies [22] to assess stool consistency in SN-2-palmitate IF related studies. In general, FF infants have harder stools compared to breast-fed (BF) infants who typically have watery to soft stools [12]. Differences in stool consistency have been mainly associated with the higher content of FA soaps in the faeces of FF infants compared to the BF ones [12]. Results from previous studies, using different stool scales to assess the effect of IF with various SN-2-palmitate content on stool consistency, have been inconsistent. Two studies found that infants receiving a high (50 and 36%, respectively) SN-2-palmitate formula had softer, less-formed stools than infants in the low (12 and 12%, respectively) SN-2-palmitate formula groups [16, 19]. In contrast, the study by Nowacki et al. 2014 [7] showed no differences between the high (39%) and the low (13%) SN-2-palmitate groups. The study by Carnielli et al. 1996 [15] showed that infants fed the high (66%) SN-2-palmitate formula had a more favourable stool consistency score than the intermediate (39%) and low (13%) SN-2-palmitate formulae. Infants fed the intermediate formula had stool consistency scores between those of the high and the low SN-2-palmitate formulae [15]. In the present study, infants consuming the 50MF formula had a mean score closer to the watery category (which is similar to the BF infants [12, 23]) and the infants consuming the VF formula had a mean score closer to the soft category, while no differences were observed for the 20MF formula vs. the VF group. The lack of difference between the 20MF formula and VF formula could be explained by the absence of hard stool reports in any of the treatment groups, which might have limited the treatment effect induced by the 19.7% SN-2-palmitate levels in 20MF formula on stool consistency. Future studies including a reference group of BF infants may provide useful and relevant insights into stool consistency of infants.

Conclusions

In summary, while the MF-based IF did not affect the concentrations of PA in stool, our studies demonstrate that increasing SN-2-palmitate in IF using bovine MF results in lower palmitate soaps, total fatty acid soaps and calcium excretion in stools in healthy, term infants. Furthermore, a favourable effect on stool consistency is also noticed with the 50MF IF. The present studies suggest a role for application of bovine MF in IF. Further research to validate these favourable effects, taking into account stereospecificity of the triglyceride, and with the inclusion of a BF reference group is warranted.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s40795-020-00365-4.

Additional file 1. Inclusion and exclusion criteria.

Additional file 2. Biochemistry analysis.

Additional file 3. Formula consumption and anthropometric data at the end of the two-week intervention periods.

Abbreviations

AISS: Amsterdam Infant Stool Scale; BF: Breastfed; CS1: Cross-over study 1; CS2: Cross-over study 2; FA: Fatty acid; FF: Formula-fed; HM: Human milk; IF: Infant formula; MF: Milk fat; PA: Palmitic acid; TAG: Triacylglycerol; VF: Vegetable fat

Acknowledgements

The authors would like to thank all the participants for their willingness to participate in the study. We also thank the study team for their valuable help in study conduct and 4Pharma for statistical support.

Authors' contributions

YM, EK, ITV, RB and PP developed the study design and protocol. YM and EK were responsible for the overall conduct of the study, quality control in the field, data interpretation and wrote the first draft of the manuscript. YM was the Principal Investigator and is the overall guarantor. ITV, MMV, RB and PP contributed to interpretation of data and provided critical comments to the manuscript. EV, AP, EM, ZM, TMK and IC contributed in the recruitment of the infants and data collection, as well as in revising the manuscript critically for important intellectual content. All authors read and approved the final manuscript.

Funding

The present study was funded by FrieslandCampina Nederland B.V. FrieslandCampina was not involved in the recruitment of participants, data collection, management and analysis.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

The protocol, information letter to the parents/caregivers and informed consent form were approved by Harokopio University's Ethics Committee. Written informed consent was obtained from parents after an explanation of the study procedures and prior to inclusion by the study paediatrician.

Consent for publication

Not applicable.

Competing interests

ITV, MMV, PP and RB are employees at FrieslandCampina. Any opinions, findings, conclusions or recommendations expressed in the current study are those of the authors and do not necessarily reflect the views of FrieslandCampina. The other authors report no conflicts of interest.

Author details

[1]Department of Nutrition and Dietetics, School of Health Science and Education, Harokopio University, Athens, Greece. [2]FrieslandCampina, Stationsplein 1, 3818 LE Amersfoort, the Netherlands. [3]Department of Endocrinology-Growth and Development, Children's Hospital P. & A. Kyriakou, Athens, Greece.

Received: 26 March 2020 Accepted: 29 July 2020
Published online: 14 September 2020

References

1. Andreas NJ, Kampmann B, Mehring L-DK. Human breast milk: a review on its composition and bioactivity. Early Hum Dev. 2015;91(11):629–35. https://doi.org/10.1016/j.earlhumdev.2015.08.013.
2. Koletzko B, Rodriguez-Palmero M, Demmelmair H, Fidler N, Jensen R, Sauerwald T. Physiological aspects of human milk lipids. Early Hum Dev. 2001;65(Suppl):S3–S18. https://doi.org/10.1016/S0378-3782(01)00204-3.
3. Miles EA, Calder PC. The influence of the position of palmitate in infant formula triacylglycerols on health outcomes. Nutr Res. 2017;44:1–8. https://doi.org/10.1016/j.nutres.2017.05.009.
4. Delplanque B, Gibson R, Koletzko B, Lapillonne A, Strandvik B. Lipid quality in infant nutrition: current knowledge and future opportunities. J Pediatr Gastroenterol Nutr. 2015;61(1):8–17. https://doi.org/10.1097/MPG.0000000000000818.
5. Straarup EM, Lauritzen L, Faerk J, Høy CE, Michaelsen KF. The stereospecific triacylglycerol structures and fatty acid profiles of human milk and infant formulas. J Pediatr Gastroenterol Nutr. 2006;42(3):293–9. https://doi.org/10.1097/01.mpg.0000214155.51036.4f.
6. Bar-Yoseph F, Lifshitz Y, Cohen T, Malard P, Xu C. SN2-palmitate reduces fatty acid excretion in Chinese formula-fed infants. J Pediatr Gastroenterol Nutr. 2016;62(2):341–7. https://doi.org/10.1097/MPG.0000000000000971.
7. Nowacki J, Lee HC, Lien R, et al. Stool fatty acid soaps, stool consistency and gastrointestinal tolerance in term infants fed infant formulas containing high sn-2 palmitate with or without oligofructose: a double-blind, randomized clinical trial. Nutr J. 2014;13(1):1–11. https://doi.org/10.1186/1475-2891-13-105.
8. Petit V, Sandoz L, Garcia-Rodenas CL. Importance of the regiospecific distribution of long-chain saturated fatty acids on gut comfort, fat and calcium absorption in infants. Prostaglandins Leukot Essent Fat Acids. 2017;121:40–51. https://doi.org/10.1016/j.plefa.2017.05.007.
9. Havlicekova Z, Jesenak M, Banovcin P, Kuchta M. Beta-palmitate - a natural component of human milk in supplemental milk formulas. Nutr J. 2016;15(1):1–8. https://doi.org/10.1186/s12937-016-0145-1.
10. Lindquist S, Hernell O. Lipid digestion and absorption in early life: an update. Curr Opin Clin Nutr Metab Care. 2010;13(3):314–20. https://doi.org/10.1097/MCO.0b013e328337bbf0.
11. Innis SM. Dietary triacylglycerol structure and its role in infant nutrition. Adv Nutr An Int Rev J. 2011;2(3):275–83. https://doi.org/10.3945/an.111.000448.
12. Quinlan PT, Lockton S, Lucas AL. The relationship between stool hardness and stool composition in breast- and formula-fed infants. J Pediatr Gastroenterol Nutr. 1995;20(1):81–90. https://doi.org/10.1097/00005176-199501000-00014.
13. Béghin L, Marchandise X, Lien E, et al. Growth, stool consistency and bone mineral content in healthy term infants fed sn-2-palmitate-enriched starter infant formula: a randomized, double-blind, multicentre clinical trial. Clin Nutr. 2018;38(3):1023–30. https://doi.org/10.1016/j.clnu.2018.05.015.
14. Carnielli VP, Luijendijk IHT, Van Goudoever JB, et al. Feeding premature newborn infants palmitic acid in amounts and stereoisomeric position similar to that of human milk: effects on fat and mineral balance. Am J Clin Nutr. 1995;61(5):1037–42. https://doi.org/10.1093/ajcn/61.5.1037.
15. Carnielli VP, Luijendijk IHT, Van Goudoever JB, et al. Structural position and amount of palmitic acid in infant formulas: effects on fat, fatty acid, and mineral balance. J Pediatr Gastroenterol Nutr. 1996;23(5):553–60. https://doi.org/10.1097/00005176-199612000-00007.
16. Kennedy K, Fewtrell MS, Morley R, et al. Double-blind, randomized trial of a synthetic triacylglycerol in formula-fed term infants: effects on stool biochemistry, stool characteristics, and bone mineralization. Am J Clin Nutr. 1999;70(5):920–7. https://doi.org/10.1093/ajcn/70.5.920.
17. López-López A, Castellote-Bargalló AI, Campoy-Folgoso C, et al. The influence of dietary palmitic acid triacylglyceride position on the fatty acid, calcium and magnesium contents of at term newborn faeces. Early Hum Dev. 2001;65(SUPPL. 2). https://doi.org/10.1016/S0378-3782(01)00210-9.
18. Lucas A, Quinlan P, Abrams S, Ryan S, Meah S, Lucas PJ. Randomised controlled trial of a synthetic triglyceride milk formula for preterm infants. Arch Dis Child Fetal Neonatal Ed. 1997;77(3):178–85. https://doi.org/10.1136/fn.77.3.F178.
19. Yao M, Lien EL, Capeding MRZ, et al. Effects of term infant formulas containing high sn-2 palmitate with and without oligofructose on stool composition, stool characteristics, and bifidogenicity. J Pediatr Gastroenterol Nutr. 2014;59(4):440–8. https://doi.org/10.1097/MPG.0000000000000443.
20. Bekkali N, Hamers SL, Reitsma JB, Van Toledo L, Benninga MA. Infant Stool Form Scale: Development and Results. J Pediatr. 2009;154(4). https://doi.org/10.1016/j.jpeds.2008.10.010.

21. Litmanovitz I, Davidson K, Eliakim A, et al. High beta-palmitate formula and bone strength in term infants: a randomized, double-blind, controlled trial. Calcif Tissue Int. 2013;92(1):35–41. https://doi.org/10.1007/s00223-012-9664-8.
22. Ghanma A, Puttemans K, Deneyer M, Benninga MA, Vandenplas Y. Amsterdam infant stool scale is more useful for assessing children who have not been toilet trained than Bristol stool scale. Acta Paediatr Int J Paediatr. 2014;103(2):2013–4. https://doi.org/10.1111/apa.12422.
23. Weaver LT, Ewing G, Taylor LC. The bowel habit of milk-fed infants. J Pediatr Gastroenterol Nutr. 1988;7(4):568–71. https://doi.org/10.1097/00005176-198807000-00015.

Publisher's Note

nutrients

MDPI

Article

A Partially Hydrolyzed Whey Infant Formula Supports Appropriate Growth: A Randomized Controlled Non-Inferiority Trial

Eva Karaglani [1], Inge Thijs-Verhoeven [2], Marjan Gros [2], Christina Chairistanidou [1], Giorgos Zervas [3], Christina Filoilia [1], Tarek-Michail Kampani [1], Vasileios Miligkos [3], Maria Matiatou [3], Stavroula Valaveri [3], Alexandros Sakellariou [1], Georgios Babilis [3], Rolf Bos [2] and Yannis Manios [1,*]

1 Department of Nutrition and Dietetics, School of Health Science and Education, Harokopio University, 70 El. Venizelou Ave, 17671 Athens, Greece; ekaragl@hua.gr (E.K.); christina.chairistanidou@gmail.com (C.C.); chfiloilia@gmail.com (C.F.); moxaxmet@otenet.gr (T.-M.K.); pediatrics.sakellariou@gmail.com (A.S.)
2 FrieslandCampina, Stationsplein 1, 3818 LE Amersfoort, The Netherlands; Inge.Thijs-Verhoeven@frieslandcampina.com (I.T.-V.); Marjan.Gros@FrieslandCampina.com (M.G.); Rolf.Bos@frieslandcampina.com (R.B.)
3 Mitera Hospital, 6 Erythrou Stavrou Str., 151 23 Athens, Greece; giorgoszervas@gmail.com (G.Z.); miligkosb@gmail.com (V.M.); dr.matiatou@gmail.com (M.M.); svalaveri@yahoo.gr (S.V.); gbampilis@yahoo.gr (G.B.)
* Correspondence: manios@hua.gr; Tel.: +30-210-9549156

Received: 4 September 2020; Accepted: 30 September 2020; Published: 6 October 2020

check for updates

Abstract: The aim of the current study was to investigate the effects of a partially hydrolyzed whey infant formula (PHF) on growth in healthy term infants as compared to a standard infant formula with intact protein (IPF). In a double-blind, non-inferiority, randomized controlled trial, a total of 163 healthy formula-fed infants, 55–80 days old, were recruited and randomly allocated to either the PHF (test) or the IPF (control) group. They were followed up for three months during which they were evaluated monthly on growth and development. In total, 21 infants discontinued the study, while 142 infants completed the study (test $n = 72$, control $n = 70$). The primary outcome was daily weight gain during the three months. Secondary outcomes included additional anthropometric indices at every timepoint over the intervention period. Daily weight gain during the three-month intervention period was similar in both groups with the lower bound of 95% confidence interval (CI) above the non-inferiority margin of −3 g/day [mean difference (95% CI) test vs. control: −0.474 (−2.460, 1.512) g/day]. Regarding secondary outcomes, i.e., infants' weight, length, head circumference, body mass index (BMI), and their Z-scores, no differences were observed between the two groups at any time point. The PHF resulted in similar infant growth outcomes as the standard IPF. Based on these results, it can be concluded that the partially hydrolyzed whey infant formula supports adequate growth in healthy term infants.

Keywords: infant formula; protein hydrolysate; growth; partially hydrolyzed formula; anthropometry

1. Introduction

Optimal feeding practices during early life are of utmost importance to support healthy growth and development in infants [1]. Human milk represents the optimum nutrition throughout infancy and is associated with several short- and long-term benefits for both the child and the mother [1–3]. However, when breastfeeding is not feasible, infant formulas (IF) are the best alternative.

Research has shown that infants who are formula-fed weigh more and have a higher risk of obesity later in life compared to breast-fed infants [4,5]. Therefore, protein sources and IF processing technologies have been modified over the past years to optimize both the quality and the quantity of proteins in IF to better suit the nutritional requirements of infants and support more optimal growth. Protein hydrolysis, i.e., where proteins are digested into smaller fragments, peptides, or amino acids, is a frequent modification in IF, particularly those designed for special medical purposes [6]. Depending on the level of hydrolysis, hydrolysates can be classified as partially or extensively hydrolyzed proteins.

Hydrolysate-based formulas have been mainly developed for cow's milk protein allergy (CMPA) management, as IF containing extensively or partially hydrolyzed proteins are suggested to reduce the risk of developing allergic manifestations during the first four to six months of life [7,8], whilst extensively hydrolyzed formulas are successfully used in symptoms' management of existing CMPA [9,10]. Furthermore, hydrolysate-based formulas are widely used for preterm infants, when breastfeeding is not available [11–13], while some studies suggest potential benefits of partially hydrolyzed formulas (PHF) in the dietary management of common functional gastrointestinal symptoms such as fussiness, reflux, and colicky symptoms in formula-fed infants [14,15].

Despite the potential benefits of hydrolyzed protein formulas on CMPA prevention or gastrointestinal tolerance, it still needs to be evaluated whether growth indices remain comparable between infants fed standard intact protein formulas (IPF) and infants fed protein hydrolysate-based IF. For this reason, new European Commission regulations [16], applying to hydrolysate-based formulas from 2021 onwards, require that the safety and suitability of each specific hydrolysate-based IF is evaluated by clinical studies.

The primary objective of the current study was to evaluate the weight gain of healthy term infants consuming a whey-based PHF compared to a standard IPF over a period of three months. The secondary objective included evaluation of additional anthropometric indices at every timepoint over the period of three months.

2. Materials and Methods

2.1. Study Design and Population

This study was a double-blind, randomized controlled trial with two study arms: The test group consuming the PHF and the control group consuming the IPF. The study was conducted in healthy, full-term, exclusively formula-fed infants. Sampling and recruitment were performed by pediatricians in two cities (Athens and Larissa) in Greece between October 2018 (first subject in) and June 2019 (last subject in), while the overall study period ended in September 2019 (last subject out). Infants were enrolled between the 55th and 80th day of age during routine visits to the pediatricians. The inclusion criteria can be found in Supplementary Methods S1. Written informed consent was obtained from the parent/legal guardian of each infant before any study procedures were initiated.

The study protocol, information letter to the parents/legal guardians, and written informed consent form were approved by Harokopio University's Ethics Committee (approval code: 62/03-07-2018). The study was conducted in accordance with the guidelines of the Declaration of Helsinki and the International Conference on Harmonization (ICH) guidelines on Good Clinical Practice (GCP) and was registered in the Netherlands Trial Registry (identifier: NL7378 (NTR7586)).

2.2. Study Procedures and Formulas

Upon inclusion in the study, subjects were randomized to one of four coded products representing the two study formulas. Randomization was performed centrally, at Harokopio University, by a designated and trained research assistant based on computer-generated schemes. For each pediatrician a distinct randomization table was created to ensure that infants recruited within one site would be appropriately randomized across treatments. Each time a pediatrician recruited an infant, the research assistant at Harokopio University was notified and she randomized the infant into one of the study

groups. Next, she informed the pediatrician which coded formula the infant would be provided with, while also arranging delivery of the appropriate formula to the infant's house.

Formulas were provided for free to the participating families during the three-month study period and were used as the sole source of nutrition for the participating infants. Formula consumption was ad libitum but a feeding table in the "Parent Information Brochure" supported a correct consumption of the study products.

The nutritional compositions of the IF used in this study are compliant to Commission Directive 2006/41/EC of 7 July 2006 amending Council Directive 91/414/EEC to include clothianidin and pethoxamid as active substances and are similar with regards to macro-nutrients, apart from the protein fraction (Table 1; for analytical composition of the two formulas see Table S2). Both IF were cow's milk based and were produced in the Netherlands by FrieslandCampina and packed in blank tins of 400 g each with a specific identification code at the bottom. All powder properties were identical between the test and control formulas. Parents/legal guardians, investigators, and study support staff were blinded to the formulas. Data analyses were performed with the study groups coded and the code was not broken until the database was locked.

Table 1. Composition of the study formulas (per 100 mL).

	Test Formula	**Control Formula**
Energy (kcal)	66	66
Intact protein (g)		1.4
Casein		0.57
Whey		0.85
Whey protein hydrolysate (g)	1.6	
Fat (g)	3.5	3.5
DHA (mg)	6.9	6.9
AA (mg)	6.9	6.9
Carbohydrates	7.0	7.0
GOS (g)	0.2	0.4
Ca (mg)	50	56
P (mg)	30	31
Na (mg)	20	23
Fe (mg)	0.78	0.77
Vitamin D (μg)	1.2	1.1

Test formula: Partially hydrolyzed whey infant formula; control formula: Intact protein formula; AA: Arachidonic acid; DHA: Docosahexaenoic acid; GOS: Galacto-oligosaccharides; Ca: Calcium; P: Phosphorus; Na: Sodium; Fe: Iron.

Once the informed consent form was obtained, baseline anthropometric measurements (weight, length, and head circumference) were performed by the pediatrician, while family demographic information, perinatal, and birth characteristics of study participants were also collected. Three follow-up visits were performed thereafter, at the following time-points: Baseline +30, +60, and +90 days, with an allowed deviation of +/−2 days. Formula intake was assessed using a paper diary, which was completed by the parent/legal guardian on seven consecutive days before the visit to the pediatrician. At each visit, the formula intake diary was collected and a clinical examination to obtain anthropometric measurements was performed by the pediatrician. Adverse events (AEs), serious adverse events (SAEs), and medication use were recorded during the follow-up visits and monitored by an independent pediatrician. No code-break requests occurred for AEs or SAEs throughout the study.

2.3. Primary and Secondary Outcome Measures

The primary outcome was weight gain (g/day) calculated as the difference in infant weight between the baseline and the 3rd follow-up visit, divided by the number of days between these visits. Secondary outcomes included other anthropometric indices assessed at each follow-up visit: Weight (g), length (cm), head circumference (cm), body mass index (BMI) (kg/m^2), and their Z-scores (based on the World Health Organization (WHO) child growth standards [17]). More details on the primary and secondary outcome measures can be found in Supplementary Methods S3.

2.4. Sample Size and Statistical Analysis

The sample size was determined according to guidelines from the American Academy of Pediatrics Task Force on Clinical Testing of Infant Formulas [18] and as described previously by Puccio et al. (2017) [19]. Specifically, the sample size calculation was based on a non-inferiority test, using a one-sided, two sample *t*-test for the comparison of weight gain at three months of intervention between treatment groups. The PASS (version 15.0.4) software was used. For the margin of non-inferiority, a weight gain of −3 g/day was determined [18]. Assuming a 2.5% significance level, a power of 80% and a standard deviation of 6.1 g/day [19], 66 infants were needed in each formula group. The expected dropout rate was estimated to be 30%, mainly because of non-compliance to the required feeding strategy, thus enrolment of 95 infants per group was planned.

The null hypothesis was that the difference in weight gain between the test and control group would be higher than −3 g/day. The alternative hypothesis of non-inferiority was that the difference in weight gain between the two groups (test minus control) would be smaller than −3 g/day.

For analysis of the primary endpoint, a one-sided statistical significance level of $\alpha = 0.025$ was used, while for the secondary endpoints, a two-sided statistical significance level of $\alpha = 0.05$ was used. No correction for multiplicity was done, because there was only one primary parameter and missing data were not imputed.

The primary endpoint (weight gain during the three-month intervention in g/day) was analyzed using an analysis of covariance (ANCOVA) model, with the study formula as a fixed factor and adjustments for multiple covariates, including baseline weight, sex, antibiotic use, birth weight, maternal pre-pregnancy BMI, father's current BMI, and average formula intake. The adjusted mean and standard error (SE) of weight gain is reported. The primary endpoint analyses were carried out in both the intention-to-treat (ITT) and per-protocol (PP) analysis sets.

The secondary endpoint analyses were also carried out in both the ITT and the PP analysis sets and were analyzed using a mixed models repeated measures (MMRM) analysis, with the study formula and visit as fixed factors, adjusting for several covariates (see primary outcome) and their interactions.

Data were analyzed independently by the statistical company OCS Life Sciences. The statistical analyses were performed using the SAS software version 9.4 or higher (SAS Institute, Cary, NC, USA).

3. Results

3.1. Study Population

A total of 163 infants were enrolled and randomized into the trial (83 test formula, 80 control formula; Figure 1). Considering that the dropout rate was much lower than 30%, the minimum number of completed subjects needed to reach statistical power ($n = 66$ per treatment group) was achieved earlier than anticipated; therefore, the recruitment was ended before 95 infants were enrolled per treatment group. Of the 163 infants recruited, 142 infants completed the study (72 test formula, 70 control formula), while 21 infants (11 test formula, 10 control formula) discontinued the study. The reasons for discontinuation for each study group can be seen in Figure 1.

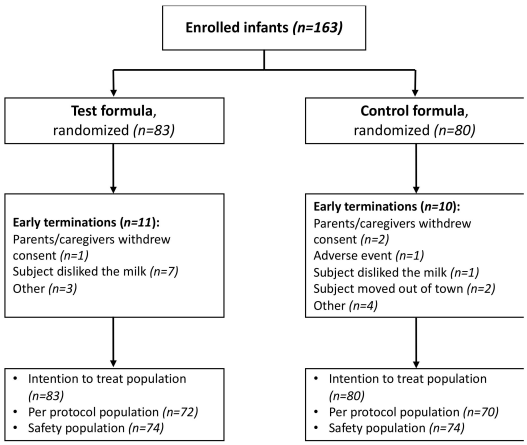

Figure 1. Study flowchart and subjects' disposition. Test formula: Partially hydrolyzed whey infant formula; control formula: Standard intact protein formula.

Demographic, perinatal, and birth characteristics were comparable between the groups, except for years of maternal education (Table 2). Baseline characteristics also did not differ between the groups except for weight at baseline, indicating that infants in the control group had a higher weight at baseline than infants in the test group (Table 2).

Table 2. Demographic, perinatal, and baseline characteristics of study infants.

	Group	
	Test (*N* = 83)	**Control (*N* = 80)**
Infant characteristics		
Age at baseline (days), mean (SD)	66.9 (7.5)	67.1 (7.5)
Gender (female), *n* (%)	41 (49.4)	39 (48.8)
Weight at baseline (g), mean (SD)	5223 (694) [1]	5443 (639)
Length at baseline (cm), mean (SD)	59.12 (2.34)	59.26 (2.94)
Head Circumference at baseline (cm), mean (SD)	38.90 (1.31)	38.74 (1.23)
Birth weight (g), mean (SD)	3206 (398)	3159 (392)
Gestational age (weeks), mean (SD)	38.3 (1.1)	38.3 (1.1)
Caesarean delivery, *n* (%)	55 (66.3)	52 (65.0)
Maternal characteristics		
Age at baseline (years), mean (SD)	32.9 (6.4)	32.7 (5.8)
Parity (primiparous), *n* (%)	41 (49.4)	34 (42.5)
BMI at baseline (kg/m^2), mean (SD)	26.03 (4.74)	27.07 (5.07)
Education, *n* (%)		
≤12 years	28 (33.7) [1]	29 (36.2)
13–16 years	53 (63.9) [1]	40 (50.0)
>16 years	2 (2.4) [1]	11 (13.8)
Smoking during pregnancy, *n* (%)	22 (26.5)	16 (20.0)
Single pregnancy, *n* (%)	75 (90.4)	72 (90.0)

[1] $p < 0.05$. Test: Partially hydrolyzed whey infant formula; control: Intact protein formula; N: Number of subjects in analysis population; SD: Standard deviation; BMI: Body mass index.

3.2. Weight Gain and Growth

In the PP population, the adjusted mean (SE) weight gain during the three-month intervention period was 24.06 (2.64) g/day for infants fed the test formula and 24.54 (2.51) g/day for those fed the control (Table 3). The mean difference (95% CI) in weight gain between groups was −0.474 (−2.460, 1.512) g/day, with the lower limit of the 95% CI above the predefined non-inferiority margin of −3 g/day, rejecting the null hypothesis and indicating a similar weight gain in the two groups. Results were similar in the ITT population.

Table 3. Weight gain of study infants from baseline to the 3rd follow-up.

Population	Group	Weight Gain (g/d) Baseline—3rd Follow-Up	Difference between Groups (Test vs. Control)		*p*-Value
		LS mean (SE)	Estimate	95% CI	
PP	Test (n = 72)	24.06 (2.635)	−0.474	−2.460, 1.512	0.637
	Control (n = 70)	24.54 (2.513)			
ITT	Test (n = 83)	23.91 (2.789)	−0.641	−2.480, 1.399	0.535
	Control (n = 80)	24.55 (2.659)			

Test: Partially hydrolyzed whey infant formula; control: Intact protein formula; PP: Per protocol; ITT: Intention to treat; CI: Confidence interval; LS mean: Least squares mean; SE: Standard error.

Regarding the secondary outcomes, in the PP population, there were no significant differences between the two groups at any follow-up visit in weight, length, head circumference, and BMI (Table S4). Furthermore, no treatment effect over time was observed for any of those indices during the three-month intervention period (Table S4). Similar results were obtained in the ITT population (Table S5). Regarding gains in weight (in g/day) from baseline to the 1st or 2nd follow-up visits, no differences were observed between the two groups (Table S6). Likewise, no differences were found for gains in length (in cm/day) between the two groups over the three-month period (from baseline to each of the three monthly follow-up assessments; Table S6). Gains in head circumference (in cm/day) were slightly lower in the test group compared to the control from baseline to the 1st follow-up visit, but no differences were observed between the two groups thereafter (from baseline to the 2nd and 3rd follow-up assessments; Table S6). All the above findings were consistent between the PP and ITT populations.

Similarly, mean weight-for-age, length-for-age, head circumference-for-age, and BMI-for-age Z-scores did not differ between the two groups at any follow-up visit. Only weight-for-length Z-scores were slightly lower in the test group compared to the control at the 1st follow-up visit, but no differences were observed between the two groups thereafter. Results were again similar in the ITT population. Figure S7 presents the relevant Z-scores of both groups during the study period in comparison with the WHO growth standards for female and male infants based on the crude (unadjusted) data. All Z-scores were tracked closely with the WHO growth standards [20].

3.3. Formula Intake and Safety Parameters

Infants in the control group had a higher weekly formula consumption (≈ +10.5%) compared to infants in the test group at all three follow-up measurements (Table 4). However, when the daily formula intake was corrected for body weight, no differences were observed between the two groups at all time points (Table 4).

Table 4. Formula intake at each follow-up visit by study group.

Daily Formula Intake by Body Weight (mL/g/d)						
	PP Population			**ITT Population**		
Study Visit	**Test LS Mean (95% CI)**	**Control LS Mean (95% CI)**	***p*-Value**	**Test LS Mean (95% CI)**	**Control LS Mean (95% CI)**	***p*-Value**
Follow-up 1	1.00 (0.96, 1.04)	1.02 (0.97, 1.06)	0.651	1.00 (0.96, 1.05)	1.01 (0.97, 1.05)	0.807
Follow-up 2	0.95 (0.90, 0.99)	0.98 (0.94, 1.02)	0.268	0.95 (0.90, 0.99)	0.98 (0.94, 1.03)	0.239
Follow-up 3	0.92 (0.89, 0.96)	0.93 (0.89, 0.97)	0.808	0.92 (0.89, 0.96)	0.93 (0.89, 0.97)	0.808
Weekly formula intake (mL)						
	PP population			**ITT population**		
	Test Median	**Control Median**	***p*-value**	**Test Median**	**Control Median**	***p*-value**
Follow-up 1	5757.5	6492.5	<0.001	5797.5	6455.0	0.001
Follow-up 2	6107.5	6880.0	<0.001	6107.5	6860.0	<.001
Follow-up 3	6420.0	7040.0	0.002	6420.0	7040.0	0.002

Test: Partially hydrolyzed whey infant formula; control: Intact protein formula; PP: Per protocol; ITT: Intention to treat; SE: Standard error.

Overall, 16 AEs occurred in the total study cohort, half of which (n = 8) occurred in the test formula group and half of which (n = 8) occurred in the control formula group. All the AEs and SAEs were unrelated to the intervention indicating no formula related risk (Table S8).

4. Discussion

The present study demonstrated a non-inferior weight gain between infants consuming a whey-based PHF and infants consuming a standard IPF during the three-month trial duration. Moreover, no differences were observed between the two groups on any growth measurements (weight, length, head circumference, and BMI), while overall growth trajectories were within the normal range based on WHO growth standards [20]. The two formulas used in the current study were similar with regards to macro-nutrients, apart from the protein fraction, and were therefore isocaloric, providing 66 kcal per 100 mL. The slight differences in galacto-oligosaccharides, which are non-digestible oligosaccharides, and some micro-nutrients could not have affected the weight gain of infants. Therefore, as hypothesized, the absence of differences on growth outcomes between the two formula groups suggests that substituting intact protein with partially hydrolyzed protein in IF is safe and supports appropriate growth in healthy infants.

Regarding the primary outcome, the current results are consistent with previous studies. In the study by Wu et al. (2018) [21], no differences were observed in daily weight gain in healthy term infants fed a PHF compared to infants fed an IPF or breast milk from enrolment to the 7th and 13th week of age. Florendo et al. (2009) [22] compared the effects of a standard non-hydrolyzed whey–casein formula to a preterm PHF for three weeks. No differences in daily weight gain were observed between the two groups during the 3-week study duration. In the German Infant Nutritional Intervention Study (GINI) [23], four different types of formulas were assessed, as well as a breast milk reference group; these formulas were either a whey PHF, an extensively hydrolyzed whey formula, an extensively hydrolyzed casein formula, or a regular IPF. Weight gain during the first four and six months of life showed no differences in infants with atopic heredity who consumed either breast milk or one of the formula groups, except for the extensively hydrolyzed casein formula which showed a transient lower weight gain. Despite the diverse study designs and IF used, it has been shown overall that no differences in weight gain were observed when healthy infants were fed either PHF or regular IPF during early infancy.

The findings of the current study on secondary outcomes, i.e., weight, length, head circumference, and BMI showed no differences between the test and control groups at all three time points. These findings are also in line with the results reported for those indices by Wu et al. (2018) [21], Florendo et al. (2009) [22], and the GINI study [23] described above. Similar findings were also reported in other studies [24,25]. Although difficult to directly compare due to methodological variations,

previous studies and current results collectively suggest that weight, length, head circumference, and BMI of infants fed either protein hydrolysate-based formulas or regular IPF do not show any differences during the first months of life.

Regarding mean Z-scores (weight-for-age, length-for-age, head circumference-for-age, weight-for-length, and BMI-for-age), the current study found no differences between the two study groups during the three-month period. Furthermore, all mean Z-scores were within the normal range based on WHO growth standards [20]. Again, consistent results have been reported by previous studies as mentioned above [21–25]. However, in the study by Menella et al. [26], Z-scores trajectories across infants aged 2.5 to 7.5 months showed significantly higher weight-for-age Z-scores in the infants fed a regular IPF compared to infants fed a PHF. Weight gain was accelerated in the former, whereas it was normative in the latter. Still, the differences observed in weight gain rates in this study could be attributed to the difference in the amount of formula consumed between the two study groups, since infants in the protein hydrolysate group consumed less formula to satiation than did regular formula-fed infants across the study period [26].

Regarding formula intake, a significant group effect was observed in the present study, with infants in the test group consuming less formula than infants in the control group at each monthly follow-up assessment. This phenomenon, also observed in the study by Menella et al. [26], could be attributed to the sensory characteristics of the two formulas, as infants may dislike the taste of protein hydrolysates, occurring due to the increased levels of free amino acids and small peptides with a bitter taste, and consequently consume less. This is further supported by the fact that the main reason for dropping out of the study in the test group was that infants disliked the test formula. Still, the overall drop-out rate was much lower than anticipated. Furthermore, it has been shown that the sooner a hydrolysate-based formula is introduced in an infant's diet, the more accepted it is by the infant [27]. Therefore, considering that infants in the present study had a mean age of 67 days at baseline, the test formula might have not been equally accepted by the infants as the control formula. Another potential explanation could be that hydrolyzed proteins have been shown to promote satiation signals and stimulate earlier meal termination in infants who consume protein hydrolysate-based formulas [28,29]. Nevertheless, the lower formula intake observed in infants consuming the test formula did not affect weight gain or other growth outcomes at any time point compared to the control formula in the current study, and supported normative growth based on WHO growth standards [20].

Among the strengths of the current study are the double-blind study design and the standardized procedure followed for data collection. Specifically, recruitment was performed by several pediatricians, but infants' growth was prospectively assessed by the same pediatrician who enrolled them in the study, during the entire study period. Still, the large number of pediatricians involved in the study could introduce some variation in the measurements performed. To ensure comparability of the anthropometric data obtained among sites, all pediatricians were trained to follow the same standardized procedures for anthropometrics, while intra- and inter-observer reliability was also periodically assessed. Another strength of the present study was that, as described in the methods section, different randomization tables were created for each pediatrician to ensure that infants would be appropriately randomized across treatments within each site.

5. Conclusions

The current study demonstrated that weight gain, as well as other growth outcomes did not differ between infants consuming the whey-based PHF and those consuming the IPF. All the Z-score indices obtained were within the normal range of WHO growth standards. Based on these results, it can be concluded that the IF with partially hydrolyzed protein supports appropriate growth in healthy term infants.

Supplementary Materials: The following are available online at http://www.mdpi.com/2072-6643/12/10/3056/s1. Methods S1: Inclusion and exclusion criteria; Table S2: Analytical composition of the study formulas (per 100 mL); Methods S3: Primary and secondary outcome measures and statistical analysis; Table S4: Weight, length, head

circumference, and BMI at each follow-up visit by study group in the PP population; Table S5: Weight, length, head circumference, and BMI at each follow-up visit by study group in the ITT population; Table S6: Gains in weight, length, and head circumference at each follow-up visit by study group; Figure S7: Anthropometric measurements expressed as Z-scores in comparison with the World Health Organization growth standards; Table S8: Overview of adverse events and serious adverse events that occurred during the trial.

Author Contributions: Conceptualization and methodology, E.K., Y.M., I.T.-V., M.G., and R.B.; investigation, E.K., Y.M., C.C., G.Z., C.F., T.-M.K., V.M., M.M., S.V., A.S., and G.B.; data curation, E.K.; writing—original draft preparation, E.K.; writing—review and editing, E.K., Y.M., I.T.-V., M.G., and R.B.; supervision, Y.M.; funding acquisition, I.T.-V., M.G., and R.B. All authors read and agreed to the published version of the manuscript.

Funding: This research was funded by FrieslandCampina Nederland B.V., which provided the infant formulas and financial support to the participating centers to conduct the study.

Acknowledgments: The authors would like to thank all the participants, as well as the study team for their valuable help with collecting data for the study.

Conflicts of Interest: I.T.-V., M.G., and R.B. are employees at FrieslandCampina and have been involved in the design of the study, the interpretation of the data, and the writing of the manuscript. Any opinions, findings, conclusions, or recommendations expressed in the current study are those of the authors and do not necessarily reflect the views of FrieslandCampina. The other authors report no conflicts of interest. The present study was funded by FrieslandCampina Nederland B.V. FrieslandCampina had no role in the recruitment of participants, in the collection, management, or analysis of data.

References

1. Innis, S.M. Impact of maternal diet on human milk composition and neurological development of infants. *Am. J. Clin. Nutr.* **2014**, *99*, 734S–741S. [CrossRef]
2. Victora, C.G.; Bahl, R.; Barros, A.J.D.; França, G.V.A.; Horton, S.; Krasevec, J.; Murch, S.; Sankar, M.J.; Walker, N.; Rollins, N.C.; et al. Breastfeeding in the 21st century: Epidemiology, mechanisms, and lifelong effect. *Lancet* **2016**, *387*, 475–490. [CrossRef]
3. Eidelman, A.I.; Schanler, R.J. Breastfeeding and the use of human milk. *Pediatrics* **2012**, *129*, e827–e841. [CrossRef]
4. Chomtho, S.; Wells, J.C.K.; Williams, J.E.; Davies, P.S.W.; Lucas, A.; Fewtrell, M.S. Infant growth and later body composition: Evidence from the 4-component model. *Am. J. Clin. Nutr.* **2008**, *87*, 1776–1784. [CrossRef]
5. Baird, J.; Fisher, D.; Lucas, P.; Kleijnen, J.; Roberts, H.; Law, C. Being big or growing fast: Systematic review of size and growth in infancy and later obesity. *Br. Med. J.* **2005**, *331*, 929. [CrossRef]
6. Vandenplas, Y.; Bhatia, J.; Shamir, R.; Agostoni, C.; Turck, D.; Staiano, A.; Szajewska, H. Hydrolyzed formulas for allergy prevention. *J. Pediatr. Gastroenterol. Nutr.* **2014**, *58*, 549–552. [CrossRef]
7. Von Berg, A.; Koletzko, S.; Grübl, A.; Filipiak-Pittroff, B.; Wichmann, H.E.; Bauer, C.P.; Reinhardt, D.; Berdel, D. The effect of hydrolyzed cow's milk formula for allergy prevention in the first year of life: The German Infant Nutritional Intervention Study, a randomized double-blind trial. *J. Allergy Clin. Immunol.* **2003**, *111*, 533–540. [CrossRef]
8. Alexander, D.D.; Cabana, M.D. Partially hydrolyzed 100% whey protein infant formula and reduced risk of atopic dermatitis: A meta-analysis. *J. Pediatr. Gastroenterol. Nutr.* **2010**, *50*, 422–430. [CrossRef]
9. Høst, A.; Koletzko, B.; Dreborg, S.; Muraro, A.; Wahn, U.; Aggett, P.; Bresson, J.L.; Hernell, O.; Lafeber, H.; Michaelsen, K.F.; et al. Dietary products used in infants for treatment and prevention of food allergy. Joint statement of the european society for paediatric allergology and clinical immunology (ESPACI) committee on hypoallergenic formulas and the european society for paediatric. *Arch. Dis. Child.* **1999**, *81*, 80–84. [CrossRef]
10. Koletzko, S.; Niggemann, B.; Arato, A.; Dias, J.A.; Heuschkel, R.; Husby, S.; Mearin, M.L.; Papadopoulou, A.; Ruemmele, F.M.; Staiano, A.; et al. Diagnostic approach and management of cow's-milk protein allergy in infants and children: Espghan gi committee practical guidelines. *J. Pediatr. Gastroenterol. Nutr.* **2012**, *55*, 221–229. [CrossRef]
11. Mihatsch, W.A.; Högel, J.; Pohlandt, F. Hydrolysed protein accelerates the gastrointestinal transport of formula in preterm infants. *Acta Paediatr. Int. J. Paediatr.* **2001**, *90*, 196–198. [CrossRef]
12. Koopman, R.; Crombach, N.; Gijsen, A.P.; Walrand, S.; Fauquant, J.; Kies, A.K.; Lemosquet, S.; Saris, W.H.M.; Boirie, Y.; Van Loon, L.J.C. Ingestion of a protein hydrolysate is accompanied by an accelerated in vivo

digestion and absorption rate when compared with its intact protein. *Am. J. Clin. Nutr.* **2009**, *90*, 106–115. [CrossRef] [PubMed]
13. Corvaglia, L.; Mariani, E.; Aceti, A.; Galletti, S.; Faldella, G. Extensively hydrolyzed protein formula reduces acid gastro-esophageal reflux in symptomatic preterm infants. *Early Hum. Dev.* **2013**, *89*, 453–455. [CrossRef] [PubMed]
14. Vandenplas, Y.; Cruchet, S.; Faure, C.; Lee, H.; Di Lorenzo, C.; Staiano, A.; Chundi, X.; Aw, M.; Gutierrez-Castrellon, P.; Asery, A.; et al. When should we use partially hydrolysed formulae for frequent gastrointestinal symptoms and allergy prevention? *Acta Paediatr.* **2014**, *103*, 689–695. [CrossRef] [PubMed]
15. Billeaud, C.; Guillet, J.; Sandler, B. Gastric emptying in infants with or without gastro-oesophageal reflux according to the type of milk. *Eur. J. Clin. Nutr.* **1990**, *44*, 577–583. [PubMed]
16. EU Commission Commission Delegated Regulation (EU) 2016/127. *Off. J. Eur. Union* **2016**, *20–30*. Available online: https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32016R0127&from=EN (accessed on 5 October 2020).
17. WHO Multicentre Growth Reference Study Group. *WHO Child Growth Standards: Length/Height-for-Age, Weight-for-Age, Weight-for-Length, Weight-for-Height and Body Mass Index-for-Age: Methods and Development*; World Health Organization: Geneva, Switzerland, 2006.
18. American Academy of Pediatrics Task Force. Clinical Testing of Infant Formulas With Respect to Nutritional Suitability for Term Infants. Available online: http://wayback.archive-it.org/7993/20170722090324/https://www.fda.gov/Food/GuidanceRegulation/GuidanceDocumentsRegulatoryInformation/InfantFormula/ucm170649.htm (accessed on 30 June 2020).
19. Puccio, G.; Alliet, P.; Cajozzo, C.; Janssens, E.; Corsello, G.; Sprenger, N.; Wernimont, S.; Egli, D.; Gosoniu, L.; Steenhout, P. Effects of infant formula with human milk oligosaccharides on growth and morbidity: A randomized multicenter trial. *J. Pediatr. Gastroenterol. Nutr.* **2017**, *64*, 624–631. [CrossRef] [PubMed]
20. WHO. WHO Child Growth Standards. *Dev. Med. Child Neurol.* **2009**, *51*, 1002. [CrossRef]
21. Wu, S.L.; Ding, D.; Fang, A.P.; Chen, P.Y.; Chen, S.; Jing, L.P.; Chen, Y.M.; Zhu, H.L. Growth, gastrointestinal tolerance and stool characteristics of healthy term infants fed an infant formula containing hydrolyzed whey protein (63%) and intact casein (37%): A randomized clinical trial. *Nutrients* **2017**, *9*, 1254. [CrossRef]
22. Florendo, K.N.; Bellflower, B.; van Zwol, A.; Cooke, R.J. Growth in preterm infants fed either a partially hydrolyzed whey or an intact casein/whey preterm infant formula. *J. Perinatol.* **2009**, *29*, 106–111. [CrossRef]
23. Rzehak, P.; Sausenthaler, S.; Koletzko, S.; Reinhardt, D.; von Berg, A.; Krämer, U.; Berdel, D.; Bollrath, C.; Grübl, A.; Cp, B.; et al. Long-term effects of feeding hydrolyzed protein infant formulas on growth extended follow-up to 10 y of age. *Am. J. Clin. Nutr.* **2009**, *89*, 1846–1856. [CrossRef] [PubMed]
24. Exl, B.M.; Deland, U.; Secretin, M.C.; Preysch, U.; Wall, M.; Shmerling, D.H. Improved general health status in an unselected infant population following an allergen-reduced dietary intervention programme: The ZUFF-STUDY-PROGRAMME - Part II: Infant growth and health status to age 6 months. *Eur. J. Nutr.* **2000**, *39*, 145–156. [CrossRef] [PubMed]
25. Wen, S.; Zhijian, W.; Mei, Z.; Qiao, X.; Yucong, C.; Meilian, D.; Hongwu, C.; Xiulan, W.; Meizhen, T.; Qianjun, L.; et al. Preventive Effect of Prenatal Education and Partially Hydrolyzed Whey Formula on Infantile Allergy: A Prospective Study. *Chin. J. Perinatol. Med.* **2015**, *18*, 904–909.
26. Mennella, J.A.; Ventura, A.K.; Beauchamp, G.K. Differential growth patterns among healthy infants fed protein hydrolysate or cow-milk formulas. *Pediatrics* **2011**, *127*, 110–118. [CrossRef] [PubMed]
27. Mennella, J.A.; Griffin, C.E.; Beauchamp, G.K. Flavor Programming during Infancy. *Pediatrics* **2004**, *113*, 840–845. [CrossRef] [PubMed]
28. Foltz, M.; Ansems, P.; Schwarz, J.; Tasker, M.C.; Lourbakos, A.; Gerhardt, C.C. Protein hydrolysates induce CCK release from enteroendocrine cells and act as partial agonists of the CCK 1 receptor. *J. Agric. Food Chem.* **2008**, *56*, 837–843. [CrossRef] [PubMed]
29. Diepvens, K.; Häberer, D.; Westerterp-Plantenga, M. Different proteins and biopeptides differently affect satiety and anorexigenic/orexigenic hormones in healthy humans. *Int. J. Obes.* **2008**, *32*, 510–518. [CrossRef]