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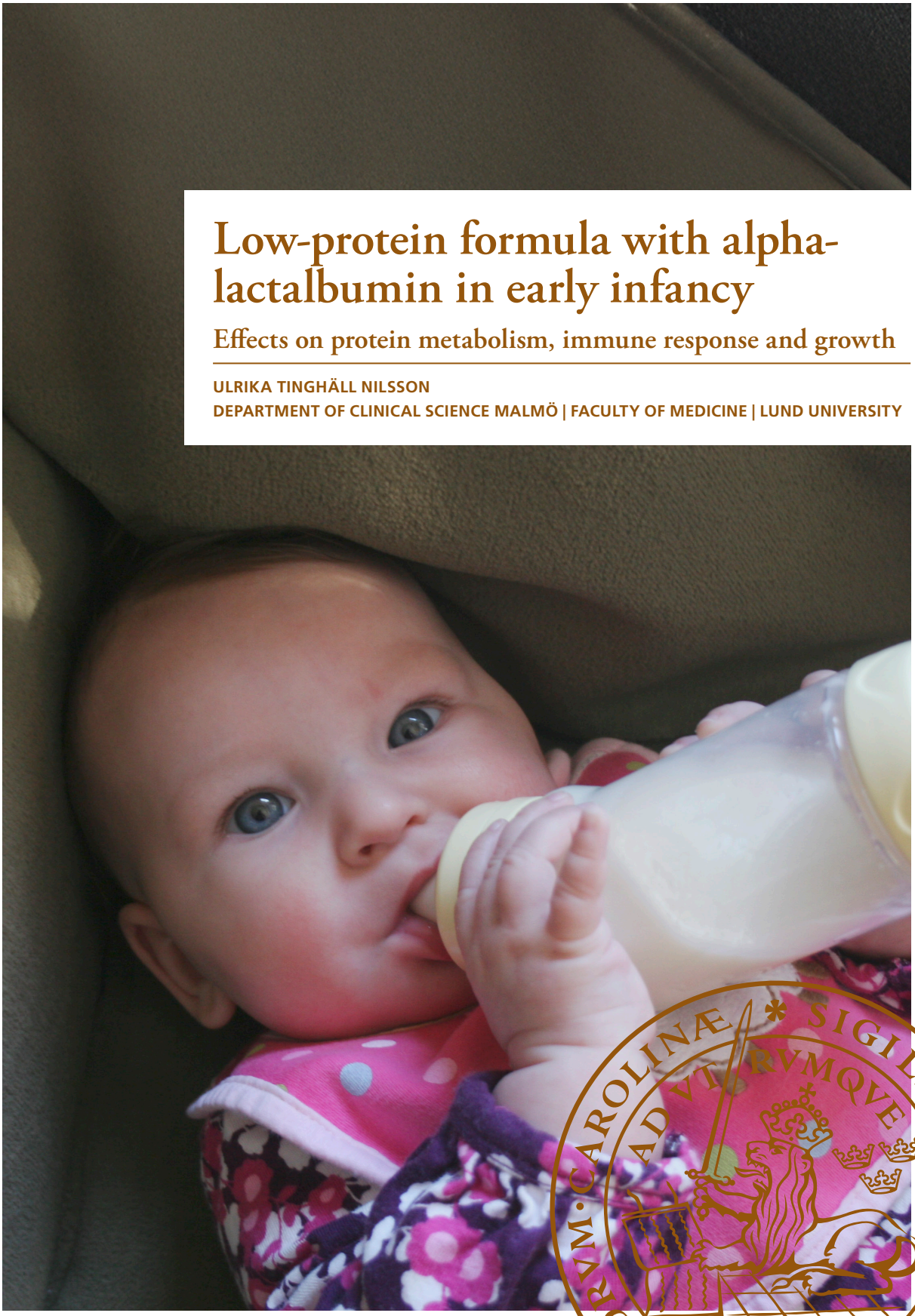

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Title and subtitle: Low-protein formula with alpha-lactalbumin in early infancy – Effects on protein metabolism, immune response and growth

Background: Formula-fed (FF) infants have higher protein intake during infancy than breastfed (BF) infants, which may lead to higher weight gain and increased risk of overweight. Enrichment with alpha-lactalbumin-enriched whey (α-lac-EW) or casein glycomacropeptide-reduced whey (CGMP-RW) to infant formula may enable further reduction of formula protein by improving the amino acid profile. BF infants have lower risk of infections, where the higher α-lac content in breast milk may promote a more favorable gut microbiota. The aims of this thesis were to evaluate if feeding low-protein (LP) infant formula with α-lac-EW or CGMP-RW would affect nutrient intake, protein metabolism, immune response and growth to be more similar to BF infants compared to feeding standard infant formula (SF), during and after intervention.

Method: In a double-blinded prospective controlled trial, 245 healthy term infants were randomized to one of two LP formulas: α-lac-EW (1.75 g protein/100 kcal, 27% α-lac) or CGMP-RW (1.76 g protein/100 kcal, 14% α-lac), or SF (2.2 g protein/100 kcal, 10% α-lac) from 2 to 6 months, with a follow-up visit at 12 months. BF infants served as the reference. Growth, dietary intake and health outcomes were assessed, and serum analysed for insulin, C-peptide, IGF-1 and cytokines, and in a subgroup of 200 infants, urea and amino acids in serum and metabolomics in serum, urine and faeces.

Results: Growth was mostly higher in FF compared to BF infants during intervention. Both LP groups had similar blood urea nitrogen (BUN) to BF infants. Serum branched chain amino acids (BCAAs) were lower in LP groups than in SF group, but still higher than in BF infants during intervention. Weight gain 6-12 months and BMI at 12 months were higher in SF than BF, but similar in LP groups and BF. Serum insulin at 12 months was higher in SF than BF group, but comparable in LP groups and BF infants. BF infants displayed a more efficient oxidation in serum and of disposal of BCAAs in urine than all FF groups. Faecal metabolome remained different in BF and FF groups throughout the intervention. Cytokines were similar in all study groups, except for interleukin-6 that was higher in all FF than in BF. Morbidity did not differ among the study groups during the intervention.

Conclusions: Feeding LP formula with α-lac-EW or CGMP-RW during early infancy was safe and resulted in normal growth and protein metabolism more similar to BF infants. The similar weight gain, BMI and serum insulin post-intervention in LP groups and BF infants, indicate that a reduced protein intake early in infancy, through lower serum BCAAs may have induced imprinting effects on insulin secretion and growth post-intervention. A reduced protein intake during early infancy could thus decrease the risk of childhood overweight. Despite improved formula protein quantity and quality, BF infants were found to oxidate and eliminate excessive amino acids and side-products more efficiently than FF infants, indicating that protein adjustment alone in infant formula do not fully replicate the metabolic effects of breast milk. The lack of effect of increased α-lac concentration in formula on inflammatory response and morbidity, as well as the different faecal metabolome in BF than in all FF groups throughout the intervention, indicate that other factors than protein influence the gut microbiota and thus probably the immune response.

Key words: alpha-lactalbumin, amino acids, protein metabolism, infant growth, infant formula, metabolomics, cytokine, infection-related morbidity, infant nutrition

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To my family

"Knowledge is power"
Sir Francis Bacon

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Tinghäll Nilsson U., Hernell O., Lönnerdal B., Lindberg Hartvigsen M., Neergaard Jacobsen L., Staudt Kvistgaard A. and Karlsland Åkeson P. Low-protein formulas with alpha-lactalbumin-enriched or glycomacropeptide-reduced-whey: Effects on growth, nutrient intake and protein metabolism during early infancy: A randomized, double-blinded controlled trial. *Nutrients 2023; 15(4).*

Paper II

Tinghäll Nilsson U., Lönnerdal B., Hernell O., Staudt Kvistgaard A., Neergard Jacobsen L. and Karlsland Åkeson P. Low-protein infant formula enriched with alpha-lactalbumin during early infancy may reduce insulin resistance at 12 months: A follow-up of a randomized controlled trial. *Nutrients 2024; 16(7).*

Paper III

He Xuan., **Tinghäll Nilsson U**., Mishchuk D, Hernell O., Lönnerdal B., Lindberg Hartvigsen M., Neergaard Jacobsen L., Staudt Kvistgaard A., Slupsky C and Karlsland Åkeson P. Impact of formula protein quantity and source on infant metabolism: Serum, urine and fecal metabolomes of a randomized controlled study. *Submitted September 2024*

Paper IV

Tinghäll Nilsson U., Hernell O., Lönnerdal B., Neergaard Jacobsen L., Salces Nuñez M, Staudt Kvistgaard A., West C. and Karlsland Åkeson P. Immunological effects of feeding low-protein infant formula with increased alpha-lactalbumin concentration during early infancy – A randomized controlled trial. *Submitted October 2024*

Abbreviations

AA	Amino acids
α-lac	Alpha-lactalbumin
α-lac-EW	Alpha-lactalbumin-enriched whey
ALFoNS	Alfa-laktalbumin och nutrition till spädbarn
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
BCAA	Branched-chain amino acid
BCKA	Branched-chain alfa-ketoacids
BF	Breastfed
BMI	Body mass index
BMIZ	BMI-for-age *z* score
BUN	Blood urea nitrogen
CHOP	Childhood obesity project trial
CGMP	Casein glycomacropeptide
CGMP-RW	Casein glycomacropeptide-reduced whey
ELISA	Enzyme-linked immunosorbent assay
FF	Formula fed
HC	Head circumference
HMOs	Human milk oligosaccharides
Hs-CRP	High-sensitivity C-reactive protein
IGF-1	Insulin-like growth factor 1
IL	Interleukin
INF-γ	Interferon gamma
ITT	Intention-to-treat

MFGM	Milk fat globule membrane
mTOR	Mammalian target of rapamycin
mTORC1	mTOR complex 1
NMR	Nuclear magnetic resonance
PP	Per-protocol
PRSL	Potential renal solute load
RCT	Randomized controlled trial
SD	Standard deviation
SF	Standard formula
TGF-β	Transforming growth factor beta
TNF-α	Tumor necrosis factor alfa
WAZ	Weight-for-age *z* score
WHO	World Health Organization

Introduction

Breast milk –the gold standard

Early life nutrition has great impact on growth, development, health and well-being, during infancy, but may also have health effects that extend into childhood, adolescence and adulthood.

Breast milk is the gold standard for infant nutrition as it contains all necessary nutrients in the right proportion to support growth and development of the infant (1, 2). If the mother cannot or choose not to breastfeed, an adapted infant formula is the only alternative during the first months of life.

During the first 6 months of life, exclusive breastfeeding is recommended by the World Health Organization (WHO) (3) and expert committees (4), and further continued breastfeeding together with a balanced diet until 2 years of age, or longer if possible, is advocated by the WHO (5).

In a world-wide perspective, less than 50% of infants are exclusively breastfed during their first 6 months of life (6). In Sweden more than 90% of the infants, at least partly, are breastfed during the first week of life. The prevalence of breastfeeding then gradually decreases, and according to the latest reports, around 65 % of infants are breastfed to some extent at 6 months of age (7, 8). To promote and support mothers to breastfeed their infants should remain the main strategy.

There are many advantages of breastfeeding compared to formula-feeding, both during the breastfeeding period but also later in life.

Breastfeeding and obesity

Breastfed infants have a reduced risk of developing childhood obesity (9-14), as well as diabetes type 2 later in life (10, 15). The protective effect of breastfeeding is important, since childhood obesity is a growing concern. In a recent report from the WHO, 37 million children in the world under the age of 5 were found to be overweight or obese (16).

Breastfeeding and morbidity and development

Breastfeeding reduces the risk of infections, mainly respiratory and gastrointestinal, and thus infant and child morbidity, and in middle-and low-income countries the risk of child mortality (6, 17-22). Furthermore, breastfeeding protects against acute otitis media during the first 2 years of life (23). Breastfeeding benefits the development and function of the immune system. Breast milk contains multiple bioactive components that are involved in the development and function of the infant´s immune system (24, 25). There is a close link between the gut microbiota and maturation and function of the immune system, where breast milk plays an important role (26-30).

Breastfeeding is also a protective factor against sudden infant death syndrome (31), and in pre-term infants, breast milk reduces the risk for necrotizing enterocolitis (6). Breastfed infants have also been reported to perform better in neurocognitive assessments than infants never breastfed (6, 32), and breastfeeding is also suggested to lower the risk of diabetes type 1 (33).

Further, mothers who breastfeed benefit from a reduced risk of breast-, and ovarian cancer (6).

Breast milk is tailored to the infant´s specific needs and improvements of infant formula composition have been achieved over the years. Although it is impossible to mimic the composition of breast milk, further optimization of infant formula must continue to try to narrow the gap between formula-fed and breastfed infants. This thesis focuses on improvement of infant formula protein content and composition.

Protein in breast milk and infant formula

A balanced diet that meets all the specific needs for optimal growth and development is essential for the rapidly growing infant. Protein is important for infant growth, where insufficient intake will impair growth while excessive intake will result in accelerated growth.

Proteins are made from smaller units, amino acids, which are linked together in specific sequences to form protein chains. Dietary proteins are digested, and the released amino acids are then used for body protein syntheses. Body proteins are essential structural component in hormones, enzymes and molecular transport systems. Nine amino acids; histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine are essential, i.e. cannot be synthesized by the cells but must be supplied from the diet. Additionally, there are two amino acids that are considered essential for infants; cysteine and tyrosine. Protein catabolism is the process where proteins are broken down to amino acids. In fact, body proteins are continuously catabolised and then resynthesized. It is important to maintain a balance within the body, so those amino acids not being

used for protein syntheses or provided in excess from the diet are degraded. Amino acids contain nitrogen which is incorporated into urea and further excreted from the body in the urine. Hence, if we have a supply of protein from the diet that exceeds the need of the body, the production of urea will increase.

During the first months of life, protein intake is solely from breast milk or infant formula. The protein concentration in breast milk changes during lactation and is highest in colostrum, around 2 g/100 ml, and then rapidly decreases to about 1 g/ 100 ml in mature milk (34). This pattern reflects the protein need of the infant, which is highest in the beginning due to the rapid growth and development and then drops during infancy. The recommended protein intake at 1 month of age is about 1.8 g/kg/day and at 6 months, 1.3 g/kg/day (35, 36).

The majority of commercially available infant formulas are made from cow´s milk. Bovine milk has a higher total protein content than breast milk, about 3.3 g/100 ml (37).

Not only total protein content, but also protein composition differs between breast milk and bovine milk.

Whey proteins are dominant in breast milk, while casein proteins are more abundant in bovine milk. The whey casein/ratio is ~ 60/40 in breast milk and ~ 20/80 in bovine milk (Figure 1) (37). The whey/casein ratio in breast milk changes during lactation, with the highest whey fraction during early lactation, about 90/10 or even higher, to provide the infant the most optimal composition, and then gradually decreases to a more even distribution during the first year of life (38, 39). Whey proteins are more easily digested and are rich in essential amino acids. Further, many human whey proteins have various bioactivities. The major whey protein in breast milk is alpha-lactalbumin (α-lac), whereas beta (β)-lactoglobulin is the major whey protein in bovine milk, not present in breast milk.

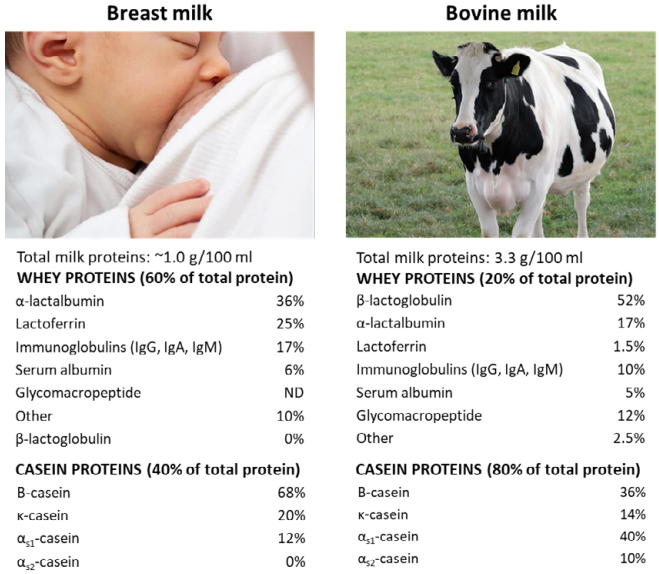


Figure 1. Protein concentration of whey and casein fractions in human and bovine milk.
Figure by author. Breast milk picture by Petr Kratochvil; https://www.publicdomainpictures.net/en/view-image.php?image=17108 CC0 Public Domain. Bovine milk picture;https://pxhere.com/en/photo/236453 CC0 Public Domain. The figure is inspired by figure 1 in the article by Layman et al (37).

The second most abundant whey protein in breast milk, lactoferrin, has iron-binding capacity, as well as antimicrobial and immunomodulatory effects (40), also in premature infants with very low birth weight, and supplementation with bovine lactoferrin is reported to protect against late-onset sepsis (41). Immunoglobulins (IgG, IgA, IgM), found in high concentrations in breast milk, are particularly important for the newborn baby as they provide passive immunization. Hence, the highest concentrations of immunoglobulins are found in colostrum (42).

The different protein compositions of breast milk and bovine milk result in different amino acid profiles. Since bovine milk is low in methionine, cysteine and tryptophan, a higher protein content in infant formula than in breast milk is required (43) to overcome the risk of shortage of essential amino acids, especially tryptophan.

Formula-fed infants have been found to have a more rapid weight gain than breastfed infants, where the higher protein intake from formula could be a reason for their different growth patterns (44-47).

A rapid weight gain, especially during the first 2 years of life, has later been linked to an increased risk for overweight and obesity in childhood, also later in life (9, 13, 48-50).

To improve infant formula composition protein concentration in infant formula has been reduced over the years based on research findings by us and others (51-61), and the whey/ casein ratio adjusted to approach the composition of breast milk. Current EU regulation states that protein concentration in commercial infant formulas shall range from 1.8 g/100 kcal to 2.5 g/100 kcal (62) in comparison to approximately 1.5 g protein/100 kcal in mature breast milk.

Early protein hypothesis

The lower protein content in breast milk than in infant formula combined with, the lower weight gain in infancy in breastfed infants and their reduced risk for childhood obesity have given rise to the "Early protein hypothesis" (63). A higher intake of protein from infant formula during infancy may induce metabolic programming of enhanced weight gain and increased risk of later overweight and obesity in formula-fed infants (64, 65). To further investigate this hypothesis a large randomized controlled trial (RCT), the European Childhood Obesity Project (CHOP) was conducted, where formula-fed infants received either higher (2.9 g (0–4 months) and 4.4 g protein/100 kcal (4–12 months)) or lower (1.77 g (0–4 months) and 2.2 g protein/100 kcal (4–12 months)) protein infant formulas and follow-on formulas, respectively. Breastfed infants were included as reference. Infants fed formula with higher protein content had higher weight gain and BMI than infants fed lower protein formula during the first 2 years of life, whereas infants fed lower protein formulas had growth rates more similar to the breastfed group (56). Infants in the higher protein formula group had higher levels of branched-chain amino acids (BCAAs; leucine, isoleucine and valine) (66), insulin growth factor 1 (IGF-1) and insulin secretion compared to infants fed the lower protein content formula (67). High protein intake induced changes in body composition with higher fat mass index compared to if a lower amount of protein was consumed (68). Long-term follow-up has shown persistently higher body mass index (BMI; kg/m^2) and increased risk for obesity in the higher protein formula group (69, 70). Conclusions from this study supported the theory that a very high intake of protein from formula results in rapid weight gain, possibly induced by increased insulin and IGF-1 levels and later risk for childhood obesity.

The mechanism (Figure 2) behind this enhanced growth could be that an excessive protein intake results in high concentrations of BCAAs, which stimulates an increased secretion of anabolic hormones, such as insulin and IGF-1. Mammalian target of rapamycin (mTOR) regulates cell growth and metabolism through complex signalling pathways, in response to nutrient intake and is activated by BCAA, especially leucine, insulin and, IGF-1. It has been proposed that persistent protein overload, through increased concentrations of BCAA, insulin and IGF-1, may lead to chronic activation of mTORC1 with enhanced growth and accumulation of fat (71).

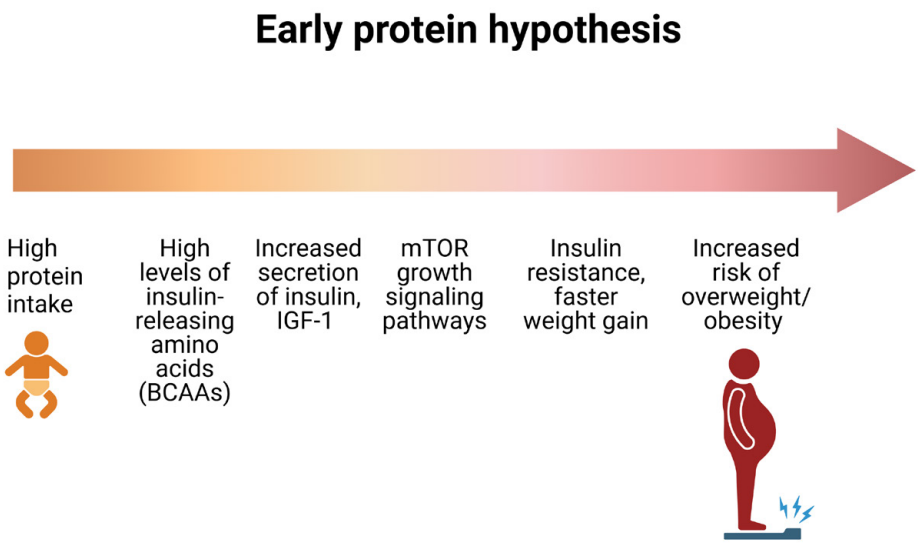


Figure 2. Early protein hypothesis. Created in BioRender. Tinghäll Nilsson, U. (2024) https://BioRender.com/v82s022

Additionally, high levels of leucine have been suggested to induce inhibition of β-oxidation of fat, resulting in higher deposition of fat and thus weight gain (66).

Despite the findings from the CHOP studies, questions have been raised about their generalizability due to the very high protein content in the higher protein formulas. Other subsequent randomized controlled trials with more moderately elevated formula protein concentrations in their standard formulas found no difference in growth during intervention compared to those fed formula with lower protein concentration (72, 73), nor were there any long-term effects on growth or body composition (74-76). Furthermore, systematic reviews conclude that current evidence for the effect of reducing protein intake on long-term outcomes such as overweight and obesity is limited (77-79). This emphasizes that additional long-term clinical studies are needed to evaluate if a reduction in protein concentration in infant formula will impact growth and metabolic status to be more similar to breastfed infants and thus result in a decreased risk of overweight and obesity in childhood.

To be able to lower the protein concentration further, to be more similar to that in breast milk, changes in the protein composition of infant formulas are required.

Alpha-lactalbumin

The most abundant whey protein in breast milk is α-lac, constituting approximately 25% of the total protein content, or about 36% of whey proteins (37, 80). Alpha-lactalbumin is a small globular protein, composed of 123 amino acids with a high proportion of essential amino acids, especially rich in tryptophan but also in lysine, BCAAs and the semi-conditional amino acid cysteine, making it a high-quality protein source (81).

Alpha-lactalbumin is essential in the milk formation process, as part of the lactose synthetase enzyme complex, which promotes the conversion of glucose and galactose into lactose in the mammary gland during lactation (82). However, α-lac can easily detach from the synthetase enzyme complex and is secreted into breast milk in high concentrations, and serves as a significant source of amino acids and contributes to ensuring a proper balance of essential amino acids in breast milk (83).

The concentration of α-lac in breast milk is highest in colostrum, with a continuously high concentration during the first month of lactation and then gradually decreases, but with a high lowest level (34, 84).

Alpha-lactalbumin is found to have a high bioavailability; the protein is digested in the small intestine into amino acids and small peptides that are effectively absorbed. Apart from nutritional functions, α-lac-derivative peptides have been suggested to have bioactive effects (37, 82, 85, 86).

In addition, α-lac has binding sites for calcium, but where one binding site also may bind trace minerals such as zinc or iron, and thus may be involved in the uptake of these trace minerals (87).

The high content of tryptophan makes α-lac important for the regulation of sleep and mood (88). Tryptophan acts as a precursor of the neurotransmitter serotonin that regulates mood, sleep and appetite. Serotonin is further metabolized to melatonin, the hormone that regulates sleep/wake cycles (37).

HAMLET (**h**uman **a**lpha-lactalbumin **m**ade **l**ethal to **t**umor cells), is a complex formed by a specific form of α-lac and oleic acid, first described to induce apoptosis and have anti-tumoral effect (86, 89). Later also shown to have bactericidal activity against several different bacteria species, especially Streptococcus species, and further if combined with antibiotics ability to enhance the effect against resistant strains (90). However, it is unknown if this complex is formed in infants in vivo (86).

Alpha-lactalbumin in infant formula

Alpha-lactalbumin is present in a much lower concentration in bovine milk, 3.5% of total protein or 17% of whey proteins, than in breast milk (37, 80) (Figure 1) and hence also in infant formula.

Increasing the α-lac concentration and reducing β-lactoglobulin will result in a whey protein composition more similar to breast milk. Since α-lac in human and bovine milk exhibits a similar and favourable amino acid composition, increasing its concentration in infant formula will enable a further reduction of protein concentration and an amino acid profile more similar to that of breast milk (37).

During infancy there is an increased need of trace minerals, such as zinc and iron. Because of lower bioavailability of these minerals in bovine milk compared to breast milk, infant formula needs to be enriched with trace minerals (91). Iron deficiency, the most frequent micronutrient deficiency globally, results in impaired growth and increased susceptibility to infections (92). However, in healthy infants with no signs of iron deficiency, an excessive iron intake could also impair growth and increase the risk for infections (93). Since α-lac can bind zinc and iron, and α-lac-derived peptides have been shown to have a high affinity for iron, increasing the α-lac concentration could be a safe and more physiological way to enable a reduction of iron and zinc in infant formula. From animal (94) and pre-clinical studies (95) α-lac has been suggested to enhance absorption of trace minerals. However, in human clinical studies, increased α-lac concentration in infant formula did not impact iron absorption in one study (96), whereas some effect was found in another study (97).

Active peptides derived from α-lac have been suggested to promote the establishment of a favourable gut microbiota composition (98-100). In two pre-clinical studies, acute diarrheal illness caused by infection with enteric pathogens was prevented by feeding a formula enriched in α-lac (98, 101). Pellegrini et al (102) reported possible bactericidal effects of α-lac peptides against several different bacteria species, and potential antiviral and various immune-modulating effects of α-lac peptides have also been described (37, 103, 104). However, no previous RCT has evaluated both infection-related morbidity and cytokine profile in infants fed α-lac-enriched infant formula in comparison to if standard infant formula or breast milk is given.

In previous studies enrichment of tryptophan to infant formulas resulted in improved sleep pattern in infants (88, 105). Increasing the α-lac concentration in infant formula will provide higher levels of tryptophan, and thus serotonin, and may be a way to improve sleep/wake rhythm in formula-fed infants.

Randomized controlled trials with α-lac-enriched formula.

Other intervention trials in healthy full-term infants evaluating formula with reduced protein content and increased α-lac concentration have been conducted, both before and after this study was designed. An overview of studies focusing on growth, and/or protein metabolism, is shown in Table 1.

Table 1. RCT with protein reduced infant formulas with increased α-lac concentration published before and after the design and start of the ALFoNS study.

Refrence	Population*, age at inclusion and setting	Intervention (duration)	Outcomes	Results
Lien (106) (2004)	SF=72, EF=62, no BF group. ≤14 days of age USA	SF: 2.25 g /100 kcal, α-lac 8%. EF: 2.15 g /100 kcal, α-lac 15%. (3 months)	Growth	No difference in growth. BUN lower in EF group.
Davis (107) (2008)	SF=43, EF=49, BF=74 ≤14 days USA	SF: 2.2 g /100 kcal, α-lac 1.3 g/L. EF: 2.08 g /100 kcal, α-lac 2.2 g/L (2 months)	Growth Protein metabolism	No difference in growth between study groups. AA similar in SF and EF. Most AA higher in EF than in BF.
Sandström (97) (2008)	SF=21, EF1=20, EF2=21, BF=34 6 ± 2 weeks Sweden	SF: 1.96 g /100 kcal, α-lac 11%. EF1: 1.96 g /100 kcal, α-lac 25%, GMP 15% EF2: 1.96 g /100 kcal, α-lac 25%, GMP 10% (4 months)	Growth Protein metabolism	Growth in EF1,EF2 more similar to BF. AA´s similar or higher than in BF.
Trabulsi (108) (2011)	SF=108, EF= 103, BF=110 ≤14 days USA	SF: 2.1 g /100 kcal, α-lac 2.2 g/L. EF: 1.9 g /100 kcal, α-lac 2.3 g/L. (3 months)	Growth Protein metabolism Metabolic markers	Growth in EF more similar to BF. Most essential AA similar in all study groups. No difference in insulin between study groups
Oropeza-Ceja (109) (2018)	SF=24, EF1=17, EF2 =18, BF=82 ≤40 days Mexico	SF: 2.18 g /100kcal, α-lac 11%. EF1: 1.43 g /100 kcal, α-lac 26%. EF2: 1.9 g /100 kcal, 26% α-lac (3 months)	Growth	Weight, weight gain similar in EF1 and BF. No differences in length or BMI between study groups.
Fleddermann (110) (2018)	SF=85, EF=82, BF=92 ≤28 days Serbia	SF: 2.2 g /100 kcal. EF: 1.89 g /100 kcal + PUFA + addition of free Trp, Phe. α-lac content not reported. (3 months)	Growth	Weight gain similar in SF, EF and higher than BF. Length gain higher in EF than SF.
Petersen (111) (2020)	SF=26, EF=26, BF=32 ≤30 days Germany	SF: 2.1 g /100 kcal, α-lac 12 % EF: 1.89 g /100 kcal, α-lac 18% (3 months)	Growth	No differences in growth between study groups.

*Number of infants that completed the intervention. SF = Standard infant formula. EF = Experimental infant formula. BF = Breastfed reference group. AA = amino acids. BUN = Blood urea nitrogen. LC-PUFA = long chain polysaturated fatty acids. Try = Tryptophan. Phe = Phenylalanine. GMP = Glycomacropeptid

These previous studies have shown that by increasing the α-lac concentration, a reduction of the total formula protein content is possible, resulting in adequate concentrations of essential amino acids and normal growth more similar to that of BF infants.

However, commercial infant formulas still have a higher protein content and a lower α-lac concentration compared to breast milk.

The ALFoNS study

In 2012 a new study was designed, the ALFoNS study, **ALF**a-laktalbumin **o**ch **N**utrition till **S**pädbarn (alpha-lactalbumin and infant nutrition), and this study became my PhD project. New whey products with higher α-lac concentrations than previously available now made it possible to further reduce the protein content and still provide sufficient supply of essential amino acids. In this study an alternative way of reducing the protein content in infant formula was also evaluated.

Casein glycomacropeptide (CGMP) is a peptide derived from milk kappa (κ)-casein and is not present in breast milk but formed in the stomach during digestion. From bovine milk κ-casein, CGMP is released during the cheese-making process when rennet is used to coagulate milk. The amino acid composition of CGMP with low concentrations of aromatic amino acids (tyrosine, tryptophan, phenylalanine), lack of cysteine and instead high contents of serine, threonine, glutamine and proline results in a unfavourable amino acid profile (112). Adding a whey product with reduced content of CGMP secures a more favourable amino acid profile and thus makes it possible to lower the total protein concentration in infant formula and still provide sufficient concentrations of essential amino acids.

In the ALFoNS study we wanted to evaluate if the addition of a new α-lac-enriched whey product or addition of a whey fraction reduced in CGMP would make it possible to safely lower protein in infant formula to concentrations slightly below current regulatory limits. Furthermore, the study design with two low-protein infant formulas but with different α-lac concentrations, allowed us to evaluate whether a decrease in total protein content or an increase in α-lactalbumin concentration in infant formula would result in outcomes more similar to those of BF infants.

Metabolomics

With standard biochemical techniques we can measure individual biomarkers or sometimes a limited panel of biomarkers to evaluate a targeted process in the body. In contrast, metabolomics is a comprehensive study of numerus metabolites, which are the products produced during metabolism, and where all these metabolites are analysed simultaneously. The metabolome refers to a more complete set of metabolites in a biological sample (113). The study of metabolomics allows a deeper

insight into cellular processes and metabolic pathways, and what effects external factors like diet will have in the body and how different diets will affect the metabolite patterns. Metabolomic analyses provide information on metabolic profiles and various interactions among metabolites far more informative than standard techniques focusing on a specific biomarker (114).

When the ALFoNS study was initiated and the analyses of metabolomics planned, knowledge about how infant formula impacted the metabolome was very limited. During the last few years, we have learned that serum, urine and faecal metabolomes are different in BF than in FF infants (115-118). However, the analysis the metabolome in all three biological samples (serum, urine and faeces) from the same human infant study population is unique, as well as the analysis of the metabolome in this way with the specific diets investigated in the ALFoNS study.

Aims

The overall aim of this thesis (I-IV) was to investigate if a reduction of protein in infant formula with the addition of α-lac-enriched whey or CGMP-reduced whey given during early infancy would result in growth, protein metabolism and immune response more similar to that of breastfed infants during and after the intervention than if feeding a standard infant formula.

Specific aims to evaluate were:

- if a modified low-protein infant formula, either with α-lac enriched whey or CGMP-reduced whey, given between 2 and 6 months of age, results in growth and protein metabolism more similar to that of breastfed infants than if feeding a standard infant formula (Paper I).
- if a modified low-protein infant formula, either with α-lac enriched whey or CGMP-reduced whey, given between 2 and 6 months of age, induces long-term effects on growth and metabolism 6 months after intervention to be more similar to that of breastfed infants than if feeding a standard infant formula (Paper II).
- if a modified low-protein infant formula either with α-lac enriched whey or CGMP-reduced whey, given between 2 and 6 months results in serum, urine and faecal metabolome during intervention more similar to breastfed infants than if feeding standard infant formula, thereby promotes the understanding of the impact of formula protein quantity and quality on infant metabolism (Paper III).
- if the addition of α-lactalbumin to low-protein infant formula in the same concentration as in breast milk during early infancy induces immune response and infection-related morbidity to be more similar to that of breastfed infants, during and after intervention (Paper IV).

Methods

Study design and population

The ALFoNS study is a randomised, double-blinded, controlled trial conducted at two sites in Sweden; Malmö/Lund and extended to Umeå in 2018. Between December 2014 and November 2019, in total 328 infants were recruited to the study, 245 formula-fed (FF) and 83 non-randomized breastfed (BF) that served as a reference group. Infants were recruited by invitation letters sent to all families with a four weeks old infant in the catchment area. Responding families were contacted by study nurses by telephone for detailed study information and screening for eligibility. Criteria for inclusion were; healthy infants, born ≥ 37 gestational weeks and appropriate for gestational age, and with birth weight 2500–4500 gram. At inclusion date, exclusively FF or exclusively BF and with the intention of parents to maintain exclusive formula-feeding or breastfeeding, respectively, until 6 months of age. Exclusion criteria were history of neonatal problems, antibiotic treatment or diseases that could interfere with normal nutrition or growth as well as born by caesarean section. The same inclusion and exclusion criteria were used for FF and BF infants.

Power calculation and sample size

Power calculation was conducted for growth, the primary outcome of the study. A sample size of 80 infants (equal distribution of boys and girls) in each group was needed to detect a difference in weight of 400 g, i.e. 0.5 SD, among the study groups at the end of the intervention (6 months of age), with 80% power at a significance level of 5%, allowing for a loss to follow-up of 20%. For a short period of time there was a higher dropout rate than expected and we decided to include additionally 8 infants to maintain power. For the analyses of amino acids and blood urea nitrogen (BUN) fewer infants were required and a subpopulation of 200 infants, 50 infants (25 girls/25 boys) from each FF group and 50 BF infants (25 girls/25 boys), were randomly selected among those having completed intervention per protocol (PP) with complete dietary records as well as blood, faecal- and urine samples at baseline, 4 and 6 months of age. For cytokine analyses, a previous power calculation in a recent RCT on cytokines in infants was used, which had shown that a sample size of 64 infants per randomized group was required to detect a difference of 0.5 SD in TNF-α concentrations between groups, with a power of 80% and a significance level of 0.05.

Intervention

Formula-fed infants were stratified by sex and assigned into random blocks of 6 or 12 using computerized randomisation to receive either standard infant formula (SF; 2.20 g protein/100 kcal, with 10% α-lac of total protein; n=83) or low-protein infant formulas with either α-lac-enriched whey (α-lac-EW; 1.75 g protein/100 kcal with 27% α-lac; n=82) or CGMP-reduced whey (CGMP-RW; 1.76 g protein/100 kcal with 14% α-lac; n=82). FF and BF infants were included in a 3:1 pattern. The protein concentration in the two low-protein formulas was slightly below the EU regulatory limit.

Compliance with the intervention was checked by study nurses at the study visits and the study formula cans were distributed on several occasions during the intervention period. In line with current Swedish national recommendations, parents were allowed to introduce tiny taste portions of complementary food from 4 months of age (119).

Group allocation was concealed to families, study staff and investigators. Unblinding of investigators occurred after all infants had completed the intervention in March 2020, and statistical analyses for the intervention period had been completed. Unblinding of families and study staff took place after the follow-up study had been completed.

Follow-up

The infants who completed the intervention study continued to be followed up to 12 months of age in the follow-up study, which was completed in October 2020. This design was in accordance with the original ALFoNS study protocol.

Study formulas

The study formulas were produced at Laiterie de Montaigu, Le Planty in France and the whey protein fractions used in the formulas, Lacprodan® ALPHA-10, Lacprodan® DI-8090 and Lacprodan® DI-8095, were provided by Arla Foods Ingredients Group P/S, Denmark. Energy and macronutrient content of study formulas are presented in Table 2A and amino acid content in Table 2B. The cans were visually identical, differing only by a code, and smell as well as taste were similar for all three study formulas. The study formulas were free of charge and distributed by the study nurses to the families.

Table 2A. Composition of study formulas

	SF	α-lac-EW	CGMP-RW
Energy (kcal/100 ml)	67.3	68.2	68.0
Whey: casein ratio	60:40	70:30	70:30
Protein (g/100ml)	1.48	1.19	1.20
Protein (g/100 kcal)	**2.20**	**1.75**	**1.76**
α-lactalbumin (%)	**10**	**27**	**14**
CGMP (%)	**9**	**19**	**-**
Carbohydrate (g)	10.1	10.1	10.2
Fat (g)	5.6	5.7	5.7

SF = standard infant formula. α-lac-EW = experimental formula with α-lactalbumin-enriched whey, CGMP-RW = experimental formula with CGMP-reduced whey.

Table 2B. Amino acid content of study formulas

	SF	α-lac-EW	CGMP-RW
Essential (mg/100 kcal)			
Histidine	56	42	45
Leucine	225	160	201
Isoleucine	127	111	96
Lysine	208	162	177
Valine	135	103	98
Phenylalanine	89	65	72
Methionine	53	36	42
Tryptophan	37	36	35
Threonine	127	117	87
Tyrosine*	82	56	66
Cysteine*	37	39	39
Nonessential (mg/100 kcal)			
Arginine	65	45	51
Alanine	93	60	77
Proline	177	118	115
Glutamic acid	439	314	344
Glycine	44	36	36
Serine	114	90	85
Aspartic acids	211	186	182

SF = standard infant formula. α-lac-EW = experimental formula with α-lactalbumin-enriched whey, CGMP-RW = experimental formula with CGMP-reduced whey. *Essential during infancy

Data collection

Infants came for monthly visits to the study centres from baseline (5–8 weeks) until 6 months of age and with a follow-up visit at 12 months. At the first visit (baseline), perinatal and background information about the infant and parents were obtained. Between the scheduled visits, families could contact the study nurses with any questions or thoughts about the study and, if a physical examination was needed, a visit could be scheduled.

Growth

Anthropometric measurements were performed at all study visits (5–8 weeks, 3, 4, 5, 6 and 12 months). Weight was measured with an accuracy of 5 g on a baby scale (Malmö/Lund: TANIT BD-815MA or UWE AIN 3, Umeå: SECA 757) whereas length (SECA 412) and head circumference (HC) (non-stretchable measuring tape (SECA 212)) were measured to an accuracy of 0.1 cm. BMI was calculated and age adjusted *z* scores for anthropometric data were calculated using international growth standards from the WHO (120, 121).

Dietary intake

Infants were fed on demand and dietary intake was documented by parents in a 3-day dietary diary every month during the intervention period between 2 and 6 months of age, and before the 12 months follow-up visit. The intake of study formula was reported in millilitre (ml) per meal and complementary feeding in ml, decilitre (dl), gram (g) or as number of tea- or table spoons. Energy and macronutrient intake from complementary food during the intervention period and from the total dietary intake at 12 months, were calculated by a dietician using a database with nutritional data from baby food manufacturers and food agencies (Dietist Net Pro®, Kost Näringsdata AB, Bromma, Sweden). When the intervention period was completed at 6 months of age, formula infants were switched to any optional follow-on formula preferred by the parents. No recommendations or restrictions regarding post-intervention nutrient intake were given to the families. In the BF group, mothers documented whenever breast milk was given, but volumes were not measured.

Gastrointestinal tolerance and morbidity

During the intervention period, parents documented, on a daily basis, information about stool consistency and frequency, and of gastrointestinal symptoms such as stomach pain, vomiting or flatulence. Two days a week, parents also documented time to fall asleep and daily crying time. Detailed information regarding morbidity

was reported both during and after intervention period, outcomes evaluated were; gastrointestinal-, and respiratory tract infections, otitis media, skin problems like eczema and rash, days of fever (≥38.0 °C), use of medication, visits to physician and admission to hospital.

Laboratory analysis

Venous blood sampling was performed at baseline, 4, 6 and 12 months visits at least two hours postprandially, and to minimize discomfort for the infant a topical anaesthetic cream was used prior to sampling. After centrifugation, the serum samples were transferred to microtubes and stored at – 80 °C at the Biobank in Lund or Umeå. Urine and faecal samples were collected at baseline, 4 and 6 months by parents at home in close proximity to the study visit, or at the study centres. Urine was collected in urinary bags and then transferred to two plastic tubes (at least 1 ml in each tube) and a minimum of 2 ml of faeces was collected from the diaper and placed in two plastic containers. If samples were collected at home, they were stored at – 20 °C until transported to study centres on freezer packs and then further stored at – 80 °C at the Biobank. When all infants had completed the intervention period, the blood, urine and faecal samples were transported on dry ice to the respective laboratory for analyses. Blood samples in the follow-up study were transported to the laboratories when all infants had completed their 12 months visit.

Insulin, C-peptide and IGF-1

Analyses of serum C-peptide and insulin were performed on a Cobas 601 instrument (Roche Diagnostics, Basel, Switzerland), and IGF-1 concentrations measured by the IDS-iSYS assay (Immunodiagnostic System Ltd, Boldon, Tyne & Wear, England). All analyses were performed at the University and Regional Laboratories in Skåne. (Paper I, II).

Hemoglobin and iron

Hemoglobin (Hb) (Paper I, II) was measured from whole blood directly after sampling at the University and Regional Laboratories in Skåne or at Norrland´s University Hospital by a Sysmex XN-10 instrument (Sysmex Corporation, Chuo-ku, Japan). Serum concentrations of iron and ferritin were analysed with a Cobas 701 instrument and Cobas 601 instrument, respectively from Roche Diagnostics, Basel, Switzerland (Paper I, II) and were conducted at University and Regional Laboratories in Skåne.

Leptin

Assessment of serum leptin concentrations was performed using ELISA (Human Leptin ELISA kit, EMD Millipore; Merck KGaA, Germany) at the Pediatric Research Laboratory at Umeå University (Paper I,II).

BUN and amino acids

For assay of BUN, a urea nitrogen colorimetric detection kit (Life Technologies Corporation, Fredrick, USA) was used and performed at the University of California, Davis, USA.

At the same laboratory, serum amino acids were analysed using ion exchange column chromatography, Hitachi 8900 (Hitachi High-Technologies Corporation, Tokyo, Japan).

Metabolome

Serum, urine and faeces metabolomes were analysed by nuclear magnetic resonance (NMR) technique at University of California , Davies, USA. Sample preparation of serum, urine and faecal material prior to analysis as well as the process of digitizing NMR data and spectral analysis have been described in detail in paper III.

Cytokines and high-sensitivity C-reactive protein

Concentrations of transforming growth factor beta 1 (TGF-β1) and TGF-β2 were measured using a TGF-β Magnetic Bead Kit (TGFBMAG-64K; EMD Millipore; Merck KGaA, Germany). Milliplex Human High Sensitivity T Cell Magnetic Beads (HSTCMAG-28SK; EMD Millipore; Merck KGaA, Germany) were used to measure tumor necrosis factor alfa (TNF-α), interferon gamma (IFN-γ), interleukin 12 (IL-12), IL-10 and IL-6. The Bio-Plex 200 instrument (Bio-Rad Laboratories, Hercules, CA) was used for all cytokine measurements. TGF-β1 and -β2 concentrations were read from a 6-point calibration curve and IL-6, IL-10, IL-12, IFN-γ and TNF-α were read from a 7-point calibration curve. The calculations were performed with Bio-Plex Manager 6.2 (Bio-Rad Laboratories, Hercules, CA).

ELISA was used for assessment of high-sensitivity C-reactive protein (hs-CRP) (human CRP Quantikine® ELISA, R&D Systems Inc., Minneapolis, USA). Cytokine and hs-CRP analyses were performed at Pediatric Research Laboratory at Umeå University.

Statistical analysis

In paper I-II and IV statistical analyses were performed using SPSS IBM statistics for Windows, version 25.0 and 28.0 (Armonk, NY, USA). For group comparison of means of normally distributed continues data one-way-analysis of variance (ANOVA), with post hoc Bonferroni was used (Paper I,II). Adjusted analysis was performed for growth data with analysis of covariance (ANCOVA), post hoc Bonferroni (Paper I,II). Non-normally continuous data were logarithmically transformed and if normality was achieved geometric means with confidence intervals are presented (Paper II), and otherwise Kruskal-Wallis test was conducted,

and data presented as median and interquartile range (Paper IV). Categorical data were analysed with Chi-square or Fishers exact test (Paper IV). Multiple linear regression (paper II) was used for assessment of association between metabolic and hormonal markers and anthropometric data. In paper III statistical analysis was conducted in R (version 4.2.2). Metabolite concentrations from metabolomics were logarithmically transformed to data normality. ANCOVA was used for group comparison and Mann-Whitney U-test was used for group comparison of non-normally distributed data. Strength of correlation was measured by Pearson´s correlation coefficient. Significance level was set to $p < 0.05$ for all analyses in all papers. Analyses were performed as intention-to-treat (ITT), but BUN, amino acids and metabolomic data were analysed in a randomized subpopulation of infants who followed the intervention per-protocol (PP).

Ethics

This study was approved by the Regional Ethical Review Board in Lund (Dnr: 2014/14) and conducted in accordance with the declaration of Helsinki. Detailed oral and written information about the study was given to the parents/ legal caregiver prior to inclusion in the study. The study was registered at ClinicalTrials.gov (NCT02410057).

Results

Study groups, drop-outs and compliance

In total, 328 infants were included to the study (Figure 3). Thirty-two FF (13%) and 10 BF infants (12%) were lost to follow-up during the intervention period. Additionally, one FF infant was excluded after being diagnosed with a genetic disease that affected growth. There were no differences among the FF groups in study withdrawal due to gastrointestinal adverse events.

The compliance to the allocated formula was high. However, one infant (SF group) switched back to pre-study formula and three infants in the SF group were prescribed cow's milk-free formula. In the BF group 6 infants needed supplementation with infant formula.

The intervention study (2–6 months) was completed by 285 infants (Figure 3), whereof 97% infants took part in the follow-up study at 12 months.

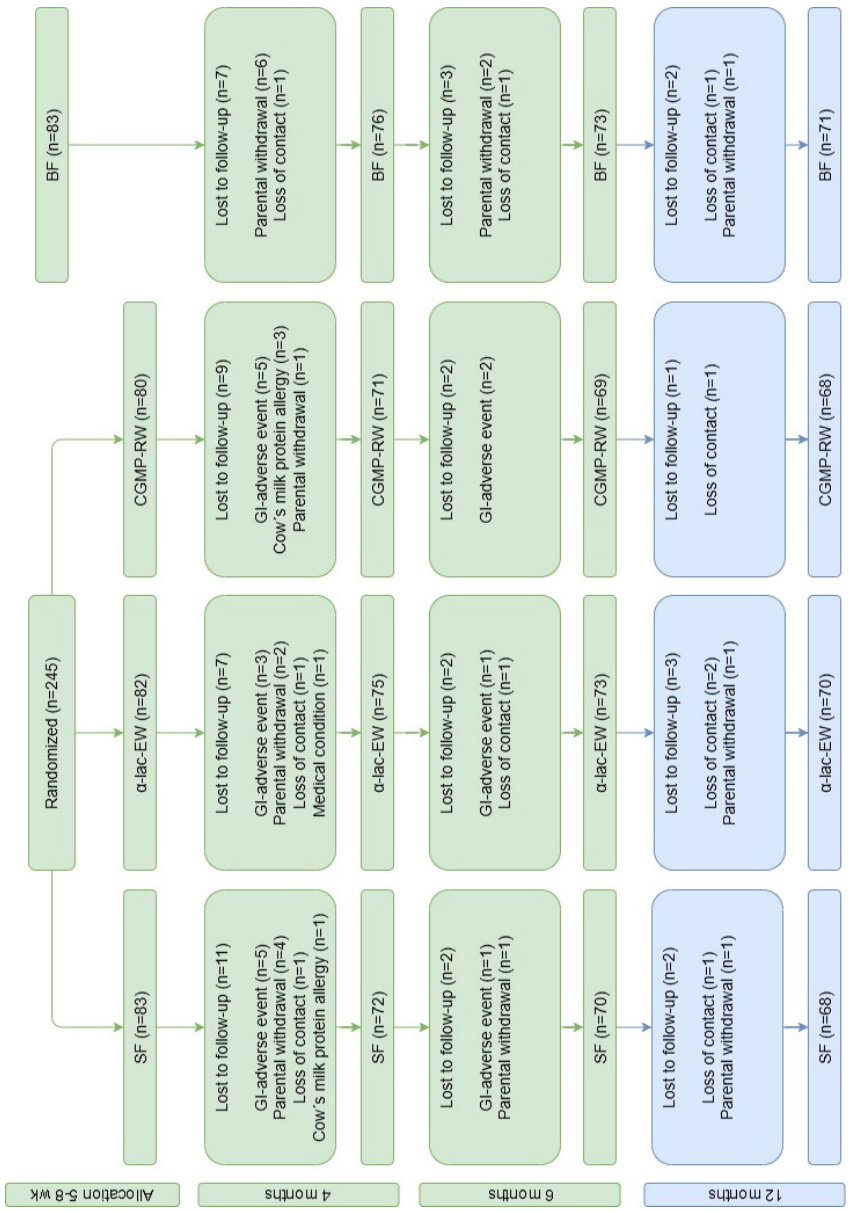


Figure 3. ALFoNS study flowchart

Few differences were found in background characteristics between BF and FF infants. Intake of probiotics (Limosilactobacillus reuteri Protectis ®) before inclusion was more common in FF infants than in BF infants (p=0.007). Mothers that were breastfeeding had a higher education level and lower BMI than mothers that gave their infants formula (p=0.002, p<0.001 respectively) (Table 3).

Table 3. Background and baseline characteristics

	SF n= 83	α-lac-EW n=81	CGMP-RW n=80	BF n=83
Gestational age (wk)	39.5 ± 1.2	39.7 ± 1.3	40.1 ± 1.2	40.0 ± 1.1
Birth weight (g)	3471 ± 452	3527 ± 440	3605 ± 443	3540 ± 424
Birth length (cm)	50.2 ± 2.2	50.1 ± 1.9	50.5 ± 1.7	50.4 ± 1.9
Birth head circumference (cm)	34.6 ± 1.2	35.0 ± 1.3	35.0 ± 1.2	35.0 ± 1.5
Age at inclusion (d)	49.3 ± 5.0	49.4 ± 4.1	49.2 ± 5.8	50.5 ± 4.5
Boy [n (%)]	43 (52)	40 (49)	40 (50)	40 (48)
Breastfed before inclusion [n (%)]	63 (76)	66 (78)	70 (88)	83 (100)
Days of breast-feeding before inclusion (n)	15.5 ± 14.6	18.0 ± 15.2	17.5 ± 13.9	50.4 ± 4.6
Probiotics* before inclusion [n (%)]	28 (34)	24 (30)	23 (29)	13 (16)
Maternal age (y)	31.5 ± 4.8	31.1 ± 4.6	31.1 ± 4.6	32.6 ± 4.2
Maternal origin [n (%)]				
Nordic	77 (93)	70 (86)	74 (93)	75 (90)
European (non-Nordic)	3 (4)	3 (10)	3 (4)	5 (4)
Non-European	3 (4)	8 (10)	3 (4)	3 (4)
Maternal BMI at enrollment (kg/m²)	27.9 ± 5.2	27.9 ± 5.6	26.1 ± 4.2	25.2 ± 3.7
Weight gain during pregnancy (kg)	13.1 ± 6.2	13.4 ± 6.6	14.7 ± 6.0	14.1 ± 5.3
Maternal higher education [n (%)] #	45 (54)	47 (58)	57 (71)	66 (80)
Maternal smoking during pregnancy [n (%)]	3 (4)	4 (5)	3 (4)	0 (0)
Paternal origin [n (%)]				
Nordic	65 (80)	68 (86)	73 (94)	65 (80)
European (non-Nordic)	6 (7)	3 (4)	2 (3)	10 (12)
Non-European	10 (12)	8 (10)	3 (4)	6 (7)
Paternal higher education [n (%)]	29 (36)	31 (40)	46 (59)	56 (69)
Paternal BMI (kg/m²)	27.0 ± 4.2	27.3 ± 5.8	26.0 ± 4.0	25.9 ± 4.5
Paternal smoking during pregnancy [n (%)]	14 (17)	11 (14)	5 (7)	9 (11)

SF = standard formula. α-lac-EW = experimental formula with α-lactalbumin-enriched whey. CGMP-RW = experimental formula with reduced CGMP whey. BF = breastfed. * Probiotics: Sempers magdroppar®, wash-out period 7 days before inclusion. # University- or higher professional education. Information about baseline characteristics available for 238 fathers.

Nutrient intake

Infants in the SF group had a higher protein intake than infants in both low-protein groups during the entire intervention (Figure 4A). The SF group also had a higher mean daily intake of study formula (ml/kg/day) than both low-protein formula groups at 3, 4 and 5 months of age (Figure 4B), this resulted in a higher energy intake from formula in the SF than in the low protein formula groups at three and four months (Figure 4C).

During the intervention, intake of complementary food was low (≤2 tablespoons/day), at 5 months 85% and 84% in FF and BF infants, respectively, and at 6 months 64% in FF infants and 69% in BF infants. There were no differences in energy and protein intake from the complementary food among the FF groups on a group level, but there was a high variation within groups and a higher energy intake from complementary feeding resulted in a correspondingly reduced intake of study formula.

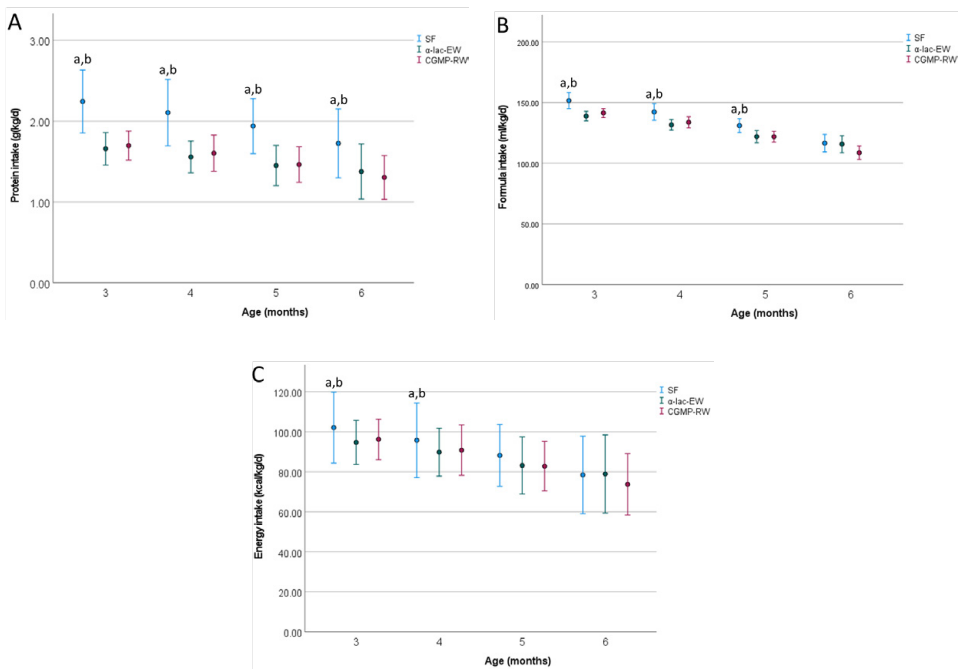


Figure 4. Nutrient intake from formula during intervention
SF = standard formula. α-lac-EW = experimental formula with α-lactalbumin-enriched whey. CGMP-RW = experimental formula with CGMP-reduced whey. BF = breastfed. Data presented as mean ± SD. Groups compared by one-way ANOVA, post hoc Bonferroni. (A) Protein intake. (B) Daily volume intake (C) Energy intake. a SF vs . α-lac-EW. b SF vs. CGMP-RW

At follow-up at 12 months, daily intake of energy, protein, carbohydrates and fat was similar in all study groups (Table 4). Since, 37% of the infants in the BF group were still partly BF, and since we did not collect information about the amount of breast milk consumed, their total nutrient intake could not be calculated.

Table 4. Daily nutrient intake at 12 months in study groups.

	SF n=66	α-lac-EW n=68	CGMP-RW n=68	p-*value*[1]	BF n=44*
Energy (kcal)	891 ± 170	892 ± 174	866 ± 157	0.61	866 ± 183
Protein (g)	28.6 ± 7.0	30.4 ± 8.0	28.8 ± 7.0	0.30	29.2 ± 7.0
Fat (g)	34.1 ± 8.4	35.0 ± 10.1	33.3 ± 8.2	0.56	33.1 ± 10.1
Carbohydrate (g)	111.2 ± 21.1	108.0 ± 22.8	106.8 ± 20.3	0.48	107.1 ± 25.0

SF = standard formula. α-lac-EW = experimental formula with α-lactalbumin-enriched whey. CGMP-RW = experimental formula with CGMP-reduced whey. BF = breastfed. Data presented as mean ± SD
[1] Formula groups compared by one-way ANOVA. * 26 partly breastfed were excluded from the analyses.

Growth

Mean weight and length did not differ among the FF groups at 4 and 6 months and all three FF groups had higher growth rates than the BF group during the intervention, except between 4 and 6 months where weight gain was similar in the α-lac-EW and BF groups (Table 5). In addition, BMI at 6 months did not differ between α-lac-EW and BF infants.

Weight gain between 6 and 12 months was similar in the low-protein formula groups and the BF group, but lower than in SF infants (Table 5). At the 12 months follow-up, no significant difference was found in mean weight or BMI among the FF groups. Mean weight did not differ significantly between the α-lac-EW and the BF groups ($p=0.05$) but was slightly higher in α-lac-EW than in BF infants when adjusted for weight at inclusion. BMI was closer to BF infants in both low-protein formula groups, whereas SF infants still had higher BMI than BF infants ($p<0.001$).

Tabel 5. Growth data at 2,4,6 and 12 months of age in all study groups.

	SF (n=83-68)	α-lac-EW (n=81-70)	CGMP-RW (n=80-68)	p-value[1] [(2)]	BF (n=83-71)
Weight (g)					
2 mo	5039 ± 585	5092 ± 566	5162 ± 604	0.41 (0.47)	5093 ± 610
4 mo	7067 ± 780 [a]	7061 ± 834 [a]	7120 ± 847 [a]	0.89 (0.80)	6685 ± 888
6 mo	8323 ± 879 [a]	8228 ± 1025 [a]	8342 ± 1009 [a]	0.76 (0.67)	7771 ± 932
12 mo	10584 ± 1222 [a]	10236 ± 1205	10315 ± 1214 [a]	0.22 (0.11)	9719 ± 962
Weight gain (g/d)					
2-6 mo	24 ± 5 [a]	23 ± 5 [a]	24 ± 5 [a]	0.59 (0.56)	20 ± 5
6-12 mo	12.5 ± 3.5 [a,b,c]	11.0 ± 2.7	10.7 ± 3.2	0.002(0.008)	10.9 ± 2.9
Length (cm)					
2 mo	56.6 ± 2.2	56.7 ± 2.0	57.1 ± 1.8	0.13 (0.25)	57.2 ± 2.0
4 mo	63.7 ± 2.6	63.7 ± 2.2	63.9 ± 2.0	0.56 (0.85)	63.4 ± 2.3
6 mo	67.9 ± 2.5	67.9 ± 2.4	67.8 ± 2.3	0.99 (0.98)	67.1 ± 2.6
12 mo	76.3 ± 2.8	76.1 ± 2.9	76.2 ± 2.4	0.91 (0.19)	75.2 ± 2.4
Length gain (mm/d)					
2-6 mo	2.5 ± 0.4 [a]	2.5 ± 0.3 [a]	2.5 ± 0.3 [a]	0.54 (0.35)	2.3 ± 0.3
6-12 mo	0.46 ± 0.09	0.45 ± 0.07	0.45 ± 0.06	0.65 (0.70)	0.45 ± 0.06
HC (cm)					
2 mo	38.7 ± 1.3	38.7 ± 1.2	38.9 ± 1.2	0.53 (0.35)	38.9 ± 1.3
4 mo	41.9 ± 1.2	41.8 ± 1.4	42.1 ± 1.5	0.27 (0.48)	41.6 ± 1.3
6 mo	43.9 ± 1.2	43.9 ± 1.6	43.9 ± 1.6	0.84 (0.71)	43.7 ± 1.5
12 mo	46.8 ± 1.2	46.7 ± 1.5	46.7 ± 1.7	0.90 (0.077)	46.5 ±1.4
HC gain (mm/d)					
2-6 mo	1.2 ± 0.2	1.2 ± 0.2	1.1 ± 0.2	0.57 (0.20)	1.1 ± 0.2
6-12 mo	0.2 ± 0.03	0.2 ± 0.03	0.2 ± 0.03	0.21 (0.18)	0.2 ± 0.03
BMI (kg/m^2)					
6 mo	18.1 ± 1.5 [a]	17.8 ± 1.6	18.1 ± 1.5 [a]	0.52 (0.44)	17.2 ± 1.3
12 mo	18.2 ± 1.6 [a]	17.6 ± 1.4	17.7 ± 1.5	0.081 (0.11)	17.1 ± 1.1

SF = standard formula. α-lac-EW = experimental formula with α-lactalbumin-enriched whey. CGMP-RW = experimental formula with CGMP-reduced whey. BF = breastfed. Data presented as mean ± SD [1] Formula groups compared by one-way ANOVA, post hoc Bonferroni. [(2)] FF groups compared by one-way ANCOVA, post-hoc Bonferroni, adjusted for baseline value of the specific outcome (if applicable), weight gain during pregnancy, gestational diabetes, maternal smoking during pregnancy, maternal and paternal BMI. HC = Head circumferences. [a] Significantly different vs. BF. [b] SF vs. α-lac-EW. [c] SF vs. CGMP-RW.

Z-scores for growth were within WHO child growth standards for all study groups during and after the intervention. Weight-for-age (WAZ) was higher in the FF groups than in the BF group during and post-intervention (Figure 5). BMI-for-age (BMIZ) was comparable to the BF group at 6 months in the α-lac-EW group, and at 12 months in both low-protein formula groups, whereas SF infants had higher BIMZ than BF infants at 6 and 12 months (Figure 5).

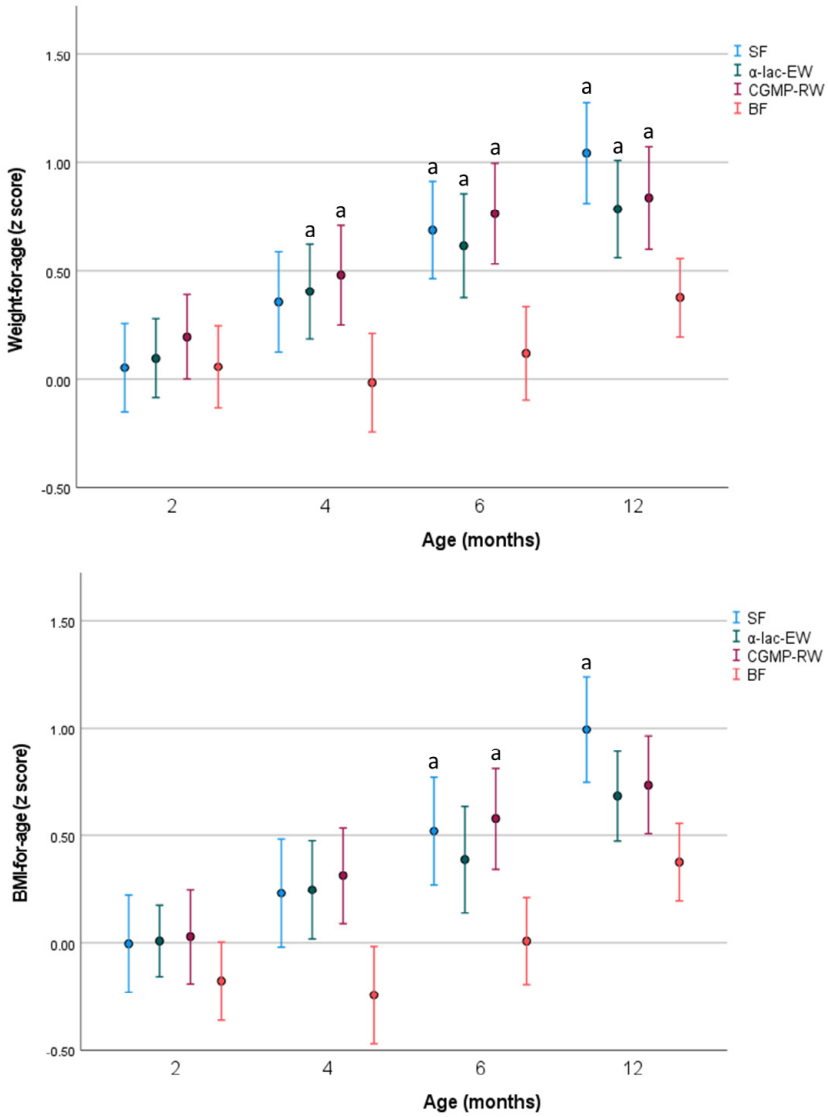


Figure 5. Mean (95% CI) of z-score weight-for-age (WAZ) and BMI-for-age (BMIZ) at 2, 4, 6 and 12 months of age

Mean (95% CI) of z-score BMI-for-age (BMIZ) and weight-for-age (WAZ). Groups compared by one-way ANOVA. SF = standard formula. α-lac-EW = experimental formula with α-lactalbumin-enriched whey. CGMP-RW = experimental formula with reduced CGMP whey. BF = Breastfed. [a] Significantly different vs. BF ($p<0.05$).

Metabolic markers

Serum concentrations of insulin, C-peptide and IGF-1 were similar in all FF groups during the intervention period, but higher than in BF infants (Table 6). However, at 12 months, serum insulin and C-peptide concentrations were similar in the low-protein formula groups and BF infants, but were still higher in the SF compared to the BF group (insulin; p<0.001), C-peptide; p=0.003). Serum IGF-1 was not significantly different among the study groups at follow-up (Table 6).

Tabel 6. Mean serum concentration of insulin, C-peptide and IGF-1 in all study groups, during the intervention and at follow-up at 12 months.

	SF (n=80-64)	α-lac-EW (n=74-69)	CGMP-RW (n=74-65)	p-value [1(2)]	BF (n=76-68)
Insulin (mIU/L)					
2 mo	11.8 ± 7.8 [a]	9.9 ± 5.2	11.5 ± 6.9 [a]	0.19 (0.42)	8.4 ± 5.4
4 mo	8.7 ± 5.2 [a]	8.1 ± 4.6 [a]	8.7 ± 6.0 [a]	0.72 (0.68)	4.9 ± 2.5
6 mo	6.8 ± 4.4 [a]	6.7 ± 5.2 [a]	7.1 ± 4.7 [a]	0.85 (0.83)	4.0 ± 2.7
12 mo	6.1 (5.0-7.4) [a]	4.8 (3.9-5.8)	4.3 (3.5-5.4)	0.056 (0.092)	3.3 (2.6-4.2)
C-Peptide (nmol/L)					
2 mo	0.69 ± 0.23 [a]	0.66 ± 0.20 [a]	0.72 ± 0.22 [a]	0.26 (0.13)	0.53 ± 0.20
4 mo	0.63 ± 0.22 [a]	0.61 ± 0.22 [a]	0.63 ± 0.24 [a]	0.90 (0.84)	0.43 ± 0.16
6 mo	0.53 ± 0.19 [a]	0.54 ± 0.25 [a]	0.55 ± 0.22 [a]	0.95 (0.97)	0.37 ± 0.17
12 mo	0.73 ± 0.37 [a,b]	0.62 ± 0.36	0.59 ± 0.24	0.035 (0.046)	0.53 ± 0.28
IGF-1 (µg/L)					
2 mo	87.2 ± 19.7	93.5 ± 18.2 [a]	88.7 ± 16.8	0.094 (0.16)	84.2 ± 20.0
4 mo	71.8 ± 22.7 [a]	71.4 ± 20.6 [a]	69.1 ± 22.1 [a]	0.74 (0.73)	55.0 ± 8.7
6 mo	60.2 ± 20.3 [a]	62.9 ± 23.0 [a]	61.1 ± 18.6 [a]	0.74 (0.92)	44.8 ± 15.2
12 mo	67.8 (62.2-73.9)	61.6 (55.6-68.3)	63.3 (57.5-69.7)	0.35 (0.86)	59.5 (53.7-65.9)

SF = standard formula. α-lac-EW = experimental formula with α-lactalbumin-enri ched whey. CGMP-RW = experimental formula with CGMP-reduced whey. BF = breastfed. Data presented as mean ± SD or as geometric mean (95%,CI). [1] Formula groups compared by one-way ANOVA, post hoc Bonferroni. [(2)] Formula groups compared by one-way ANCOVA, post hoc Bonferroni, adjusted for baseline value (if applicalbe), weight gain during pregnancy, smoking, gestational diabetes, BMI mother and father. [a] Significantly different vs. BF (p<0.005). [b] SF vs. CGMP-RW.

Serum insulin at 12 months, but not at 6 months, was associated with weight gain between 6 to 12 months of age (β coefficient = 0.7 (CI: 0,2,1.1), p = 0.005).

Protein metabolism

During the intervention, BUN was similar in the two low-protein formula groups and the BF group, but lower than in the SF group (Figure 6).

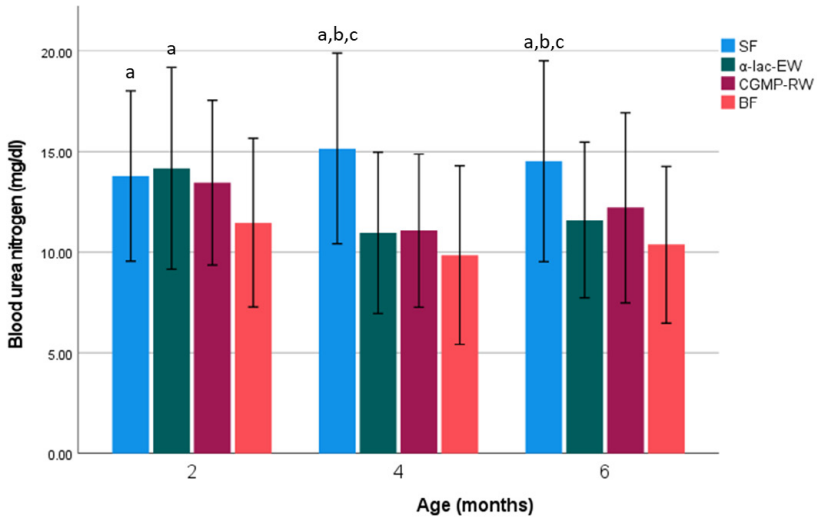


Figure 6. Blood urea nitrogen concentration at 2, 4 and 6 months of age in all study groups. SF = standard formula. α-lac-EW = experimental formula with α-lactalbumin-enriched whey. CGMP-RW = experimental formula with reduced CGMP whey. BF = breastfed. Data presented as mean ± SD. Groups compared by one-way ANOVA, post hoc Bonferroni. [a] Significantly different vs. BF. [b] SF vs. α-lac-EW. [c] SF vs. CGMP-RW.

Serum concentrations of tryptophan were similar in all study groups during the intervention, except at 6 months when it was higher in the CGMP-RW group than in BF infants (Figure 7). Serum leucine was similar in α-lac-EW and BF group, but lower than in the CGMP-RW and SF groups. During the intervention, serum valine was higher in the SF group than in all other study groups, and lower in CGMP-RW infants than in the α-lac-EW group. Serum isoleucine were found to be higher in SF infants than in the CGMP-RW and BF groups, and lower in CGMP-RW than in α-lac-EW infants (Figure 7).

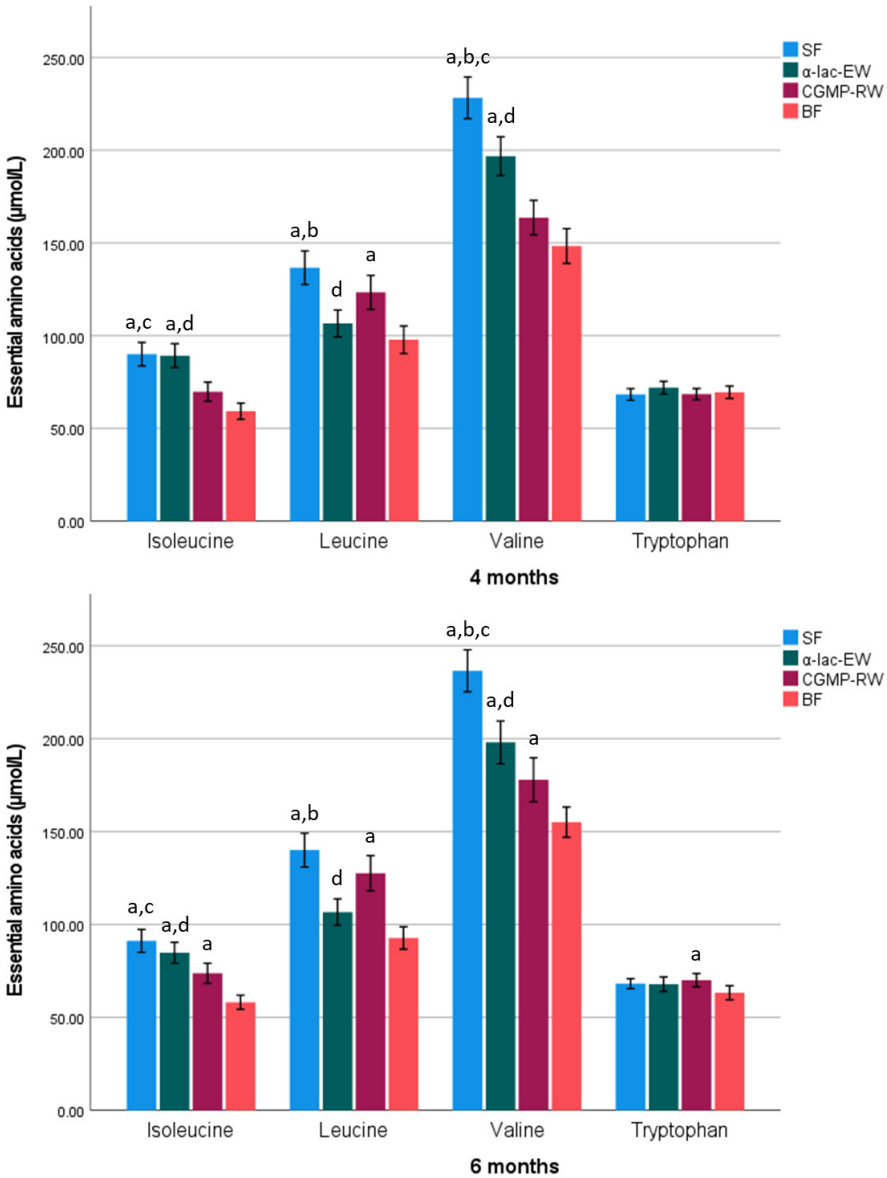


Figure 7. Serum concentration of isoleucine, leucine, valine and tryptophan at 4 and 6 months. SF = standard formula. α-lac-EW = experimental formula with α-lactalbumin-enriched whey. CGMP-RW = experimental formula with reduced CGMP whey. BF = breastfed. Data presented as mean ± SD. Groups compared by one-way ANOVA, post hoc Bonferroni. [a] Significantly different vs. BF. [b] SF vs. α-lac-EW. [c] SF vs. CGMP-RW. [d] α-lac-EW vs. CGMP-RW.

Mean total BCAAs (isoleucine (ile), leucine (leu) and valine (val)) was higher in FF infants than in BF infants, but lower in the α-lac-EW and CGMP-RW groups than in the SF group during the intervention (Figure 8).

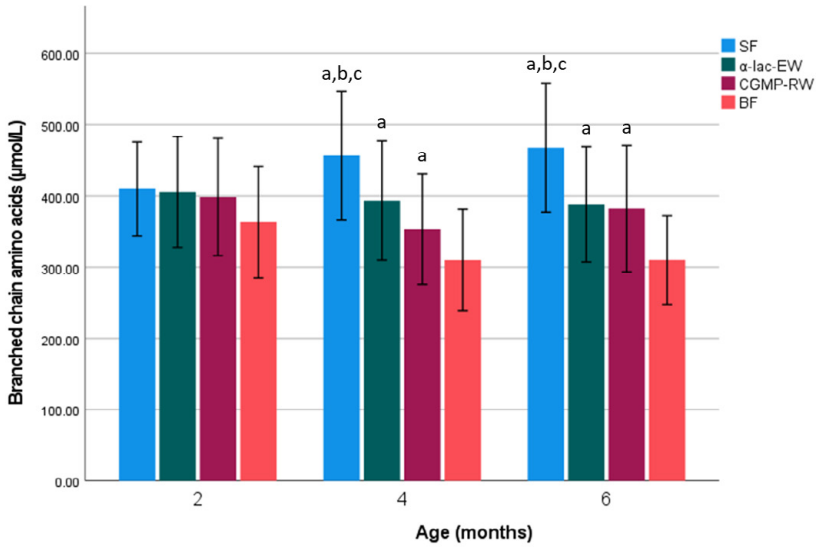


Figure 8. Serum concentration of total BCAAs (isoleucine, leucine, valine) at 2, 4 and 6 months. SF = standard formula. α-lac-EW = experimental formula with α-lactalbumin-enriched whey. CGMP-RW = experimental formula with reduced CGMP whey. BF = breastfed. Data presented as mean ± SD. Groups compared by one-way ANOVA, post hoc Bonferroni. [a] Significantly different vs. BF. [b] SF vs. α-lac-EW. [c] SF vs. CGMP-RW.

Metabolome

At inclusion, serum, urine and faecal metabolomes did not differ between the FF groups. During intervention serum and urine metabolomes were both affected by the protein concentration, as well as by the different protein compositions of the formulas. The metabolomes in serum, urine and faeces differed between the BF group and FF groups from inclusion and throughout the intervention period (Figure 9). We did not observe any significant differences in total complementary food intake among the study groups, but intake of type of complementary food varied within the groups, which also influenced the serum, urine and faecal metabolomes at 6 months (Figure 9).

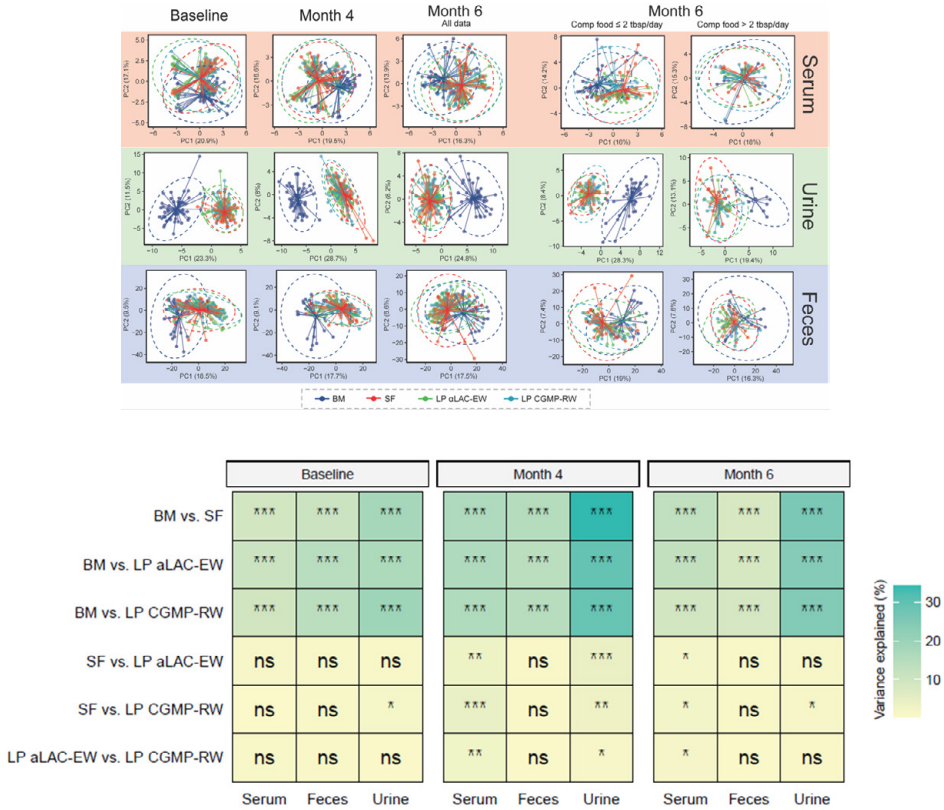


Figure 9. Serum, urine and faecal metabolomes during intervention period
SF = standard formula. LP aLAC-EW = experimental formula with α-lactalbumin-enriched whey. CGMP-RW = experimental formula with reduced CGMP whey. BM = breastfed. Ns = not significant different. To illustrate the impact of complementary food intake on the infant metabolome, data at 6 months was further stratified based on complementary food intake (≤ or > 2 tablespoon per day).

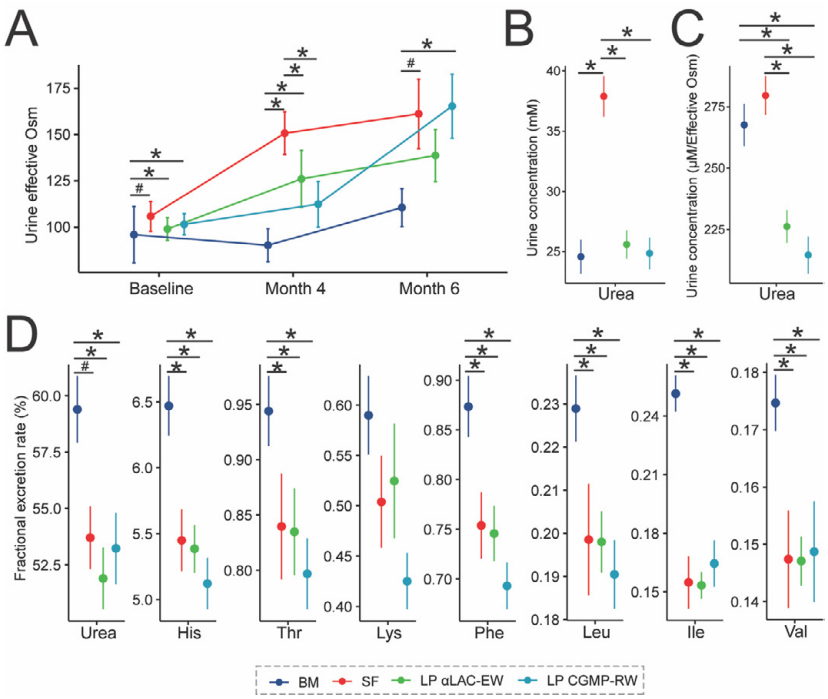
Serum metabolome

BF infants had a serum metabolome characterized by elevated levels of ketogenic metabolites, TCA intermediates and short-chain fatty acids. FF infants had higher concentrations of urea and essential amino acids, i.e. from protein catabolism.

Urine metabolome

FF infants had a more concentrated urine with higher urine effective osmolarity in comparison to BF infants (Figure 10 A), and where hydration status impacted on urea secretion (Figure 10 B,C). However, at 4 months, infants in both low-protein formula groups had lower urine effective osmolarity compared to SF infants (Figure

10 A). In all FF infants, catabolites from essential amino acids, especially BCAAs were more concentrated in urine than in serum. FF infants had a lower excretion rate of essential amino acids and urea in urine than BF infants (Figure 10 D).



Figur 10. Impact of diet on urine metabolome
SF = standard formula. LP aLAC-EW = experimental formula with α-lactalbumin-enriched whey. CGMP-RW = experimental formula with reduced CGMP whey. BM = breastfed. Data presented as mean ± SEM. (A) Urine effective osmolarity. Urin urea concentrations, uncorrcted (B) and corrected (C) for hydration status. Effective osmolarity was determined by subtracting the molar contribution of urinary urea from measured osmolality. (D) Fractional excreation rate of urea and essential amino acids. The fractional excretion rate (%) was calculated using the formula: ([urine metabolite] × [serum creatinine] / [serum metabolite] / [urine creatinine])× 100%.

Faecal metabolome

The faecal metabolome differed between the BF and FF infants at baseline and during the entire intervention period. No differences were found among FF infants at any time point (Figure 9). Stools from BF infants consisted of metabolites from microbial carbohydrate utilization, whereas stools from FF infants consisted of metabolites from microbial fermentation of protein.

Dietary effects on BCAA catabolism

In the first step of BCAA catabolism, that constitutes the reversible conversion of leucine, isoleucine and valine to BCKAs (Figure 11), infants in the α-lac-EW group had lower levels of serum BCKA catabolites related to the leucine catabolism than the SF and CGMP-RW groups, whereas the CGMP-RW group had the lowest levels of BCKA catabolites related to isoleucine and valine catabolism (12 A). BF infants had higher ratios of BCKA to BCAA than all FF groups (Figure 12 B).

BF infants had higher urinary levels of leucine and valine catabolites (Figure 12 C) as well as higher fractional excretion rates of these catabolites in urine than FF infants (Figure 12 D), both at baseline and during the intervention.

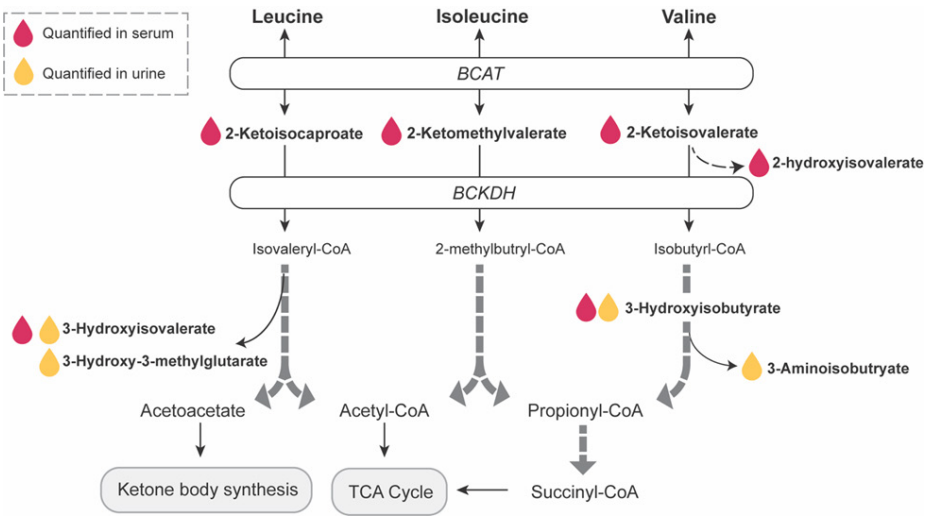


Figure 11. BCAA catabolism pathways
BCAT=branched-chain amino transferase. BCKDH =branched-chain α-ketoacid dehydrogenase. TCA= tricarboxylic acid.

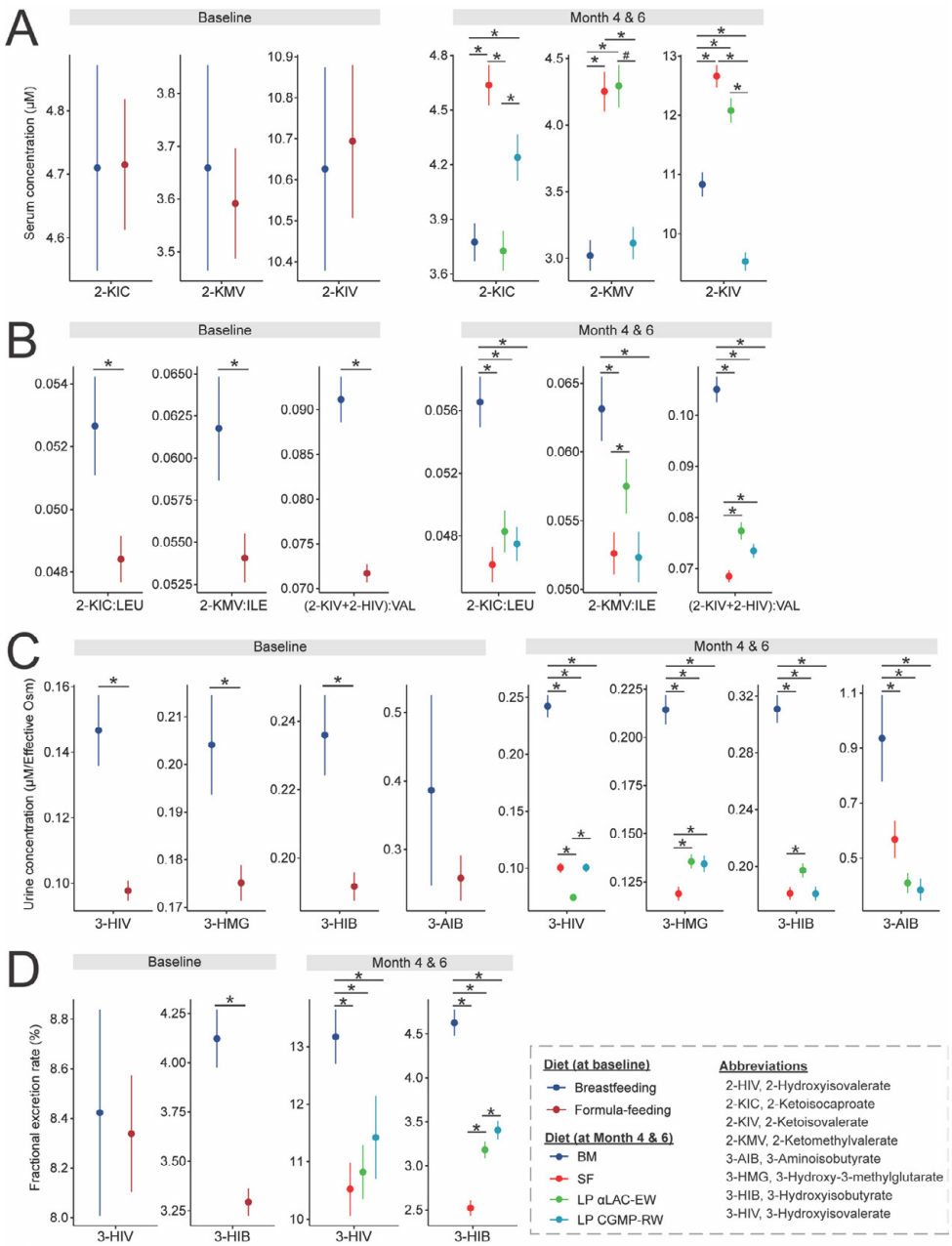


Figure 12. Dietary effects on markers of BCAA catabolism.

SF=standard formula. LP aLAC-EW = experimental formula with α-lactalbumin-enriched whey. CGMP-RW = experimental formula with reduced CGMP whey. BM = breastfed. Data presented as mean ± SEM. (A) Serum levels of the branched-chain α-ketoacids (BCKA) 2-ketoisocaproate, 2-ketomethylvalerate, 2-ketoisovalerate. (B) Ratio of BCKA and BCAA in serum. (C) Concentrations of urinary BCAA catabolite. (D). Fractional excretion rates of BCAA catabolites in urine.

Gastrointestinal tolerance, time to fall asleep and iron status

All study formulas were well tolerated with no differences among the FF groups in terms of gastrointestinal symptoms such as abdominal pain, flatulence or vomiting. There were no differences in frequency of hard stools or diarrhoea among the FF groups (Figure 13). BF infants had a higher number of stools per day (2.0 stools/day) compared to 1.1-1.3 stool/day in the formula groups.

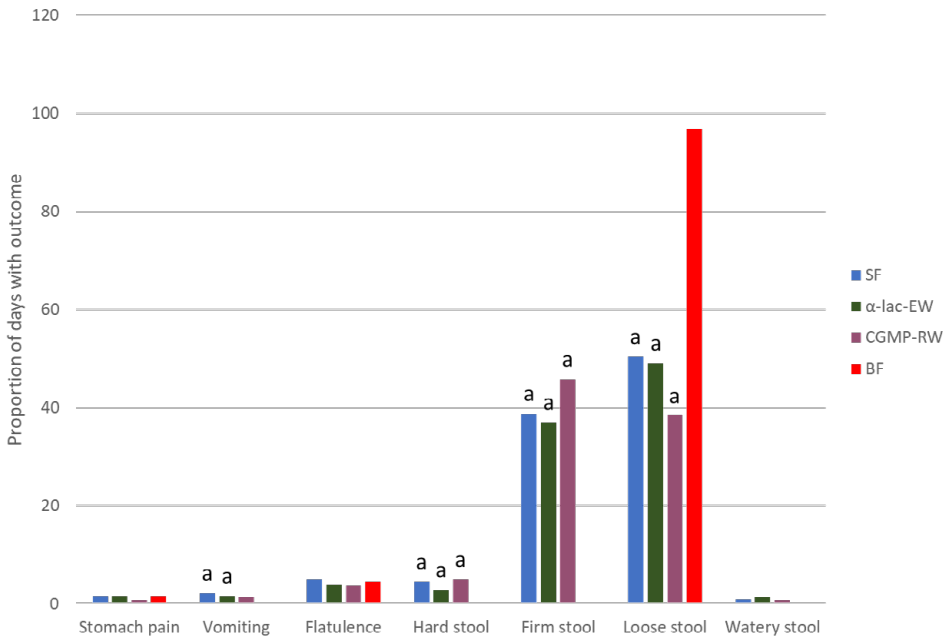


Figure 13. Proportion of days with gastrointestinal symptoms and different stool concistency. SF = standard formula. α-lac-EW = experimental formula with α-lactalbumin-enriched whey. CGMP-RW = experimental formula with reduced CGMP whey. BF = breastfed. [a] Significantly different vs. BF.

For most infants, it took less than 15 minutes to fall asleep, with no difference among study groups.

During the intervention, hemoglobin was similar in all study groups and serum iron concentration similar in all FF groups. S-ferritin did not differ among FF groups except at 4 months where serum ferritin concentration was higher in CGMP-RW than in the SF group. No infant had any signs of iron depletion.

Immune response

Infection-related morbidity and treatment

The cumulative incidence (the proportion of participants presenting a specific outcome at least once during the time period) of infection-related morbidity and treatment outcomes during and after the intervention period are shown in Figure 14. The cumulative use of antipyretics was found to be higher in all three FF groups than in the BF group (α-lac-EW vs. BF p=0.008, CGMP-RW vs. BF p=0.028 and SF vs. BF p<0.001, respectively) during the first 6 months of life. However, the longitudinal prevalence (the proportion of days with a specific outcome) of fever did not differ among the study groups. The cumulative use of antibiotics was low in all study groups, and significantly lower in the α-lac-EW than in the CGMP-RW group (p=0.008) during the intervention. A trend towards a lower cumulative incidence of acute otitis media was observed in the α-lac-EW compared to the CGMP-RW group (p=0.053) during the same time period. There was a higher incidence of otitis media in α-lac-EW than in BF infants (p=0.046) between 6 and 12 months, as was the use of antibiotics (p=0.016). No other differences in infection-related morbidity or treatment outcomes were found during or after the intervention.

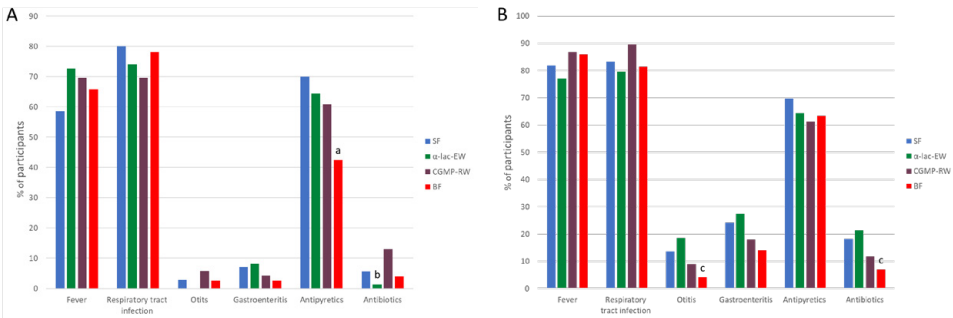


Figure 14. Infection-related morbidity and treatment during intervention (A) and post-intervention (B)

Cumulative incidence (percentage of infants with each outcome) of infection-related symptoms and treatment during intervention (2 to 6 months) and in post-intervention period (6-12 months) [a] BF vs. FF groups. [b] CGMP-RW vs. α-lac EW . [c] α-lac EW vs. BF.

Cytokines and hsCRP

We found no significant differences in any of the cytokine concentrations (TNF-α, INF-γ, IL-6, IL-10, IL-12, TGF-β1, TGF-β2) among the FF groups at baseline or during the intervention. In Table 7, serum concentrations of two proinflammatory cytokines, TNF-α and IL-6**,** and two with anti-inflammatory capacity (TGF-β1,

TGF-β2) are reported. Serum IL-6 was similar in all study groups at baseline, but then increased significantly in all three FF groups during intervention but remained low in the BF group.

Serum hsCRP was normal, < 3.0 mg/ml, and similar in all study groups during the intervention period.

Table 7. Cytokine concentrations at 2, 4 and 6 months of age in all study groups

	SF (n=71-65)	α-lac-EW (n=66-65)	CGMP-RW (n=71-64)	p-value	BF (n=67-61)
TNF-α (pg/ml)					
2 mo	26.1 (21.7;31.0)	29.1 (24.8;35.4)	26.1 (22.4;33.0)	0.065	26.7 (23.4;33.5)
4 mo	25.8 (19.7;31.9)	25.6 (21.2;30.6)	25.9 (21.1;35.0)	0.52	26.6 (21.7;33.4)
6 mo	26.5 (21.4-31.1)	24.3 (19.0;29.6)	23.9 (18.7;29.2)	0.17	25.6 (20.9;31.3)
IL-6 (pg/ml)					
2 mo	0.4 (0.1;6.1)	0.1 (0.1;3.4)	0.5 (0.1;4.8)	0.53	0.1 (0.1;4.0)
4 mo	10.5 (2.1;24.5) [a]	4.3 (0.1;21.2) [a]	7.7 (2.1;20.1) [a]	0.28	0.1 (0.1;2.1)
6 mo	13.4 (2.6;37.0) [a]	20.2 (2.2;66.5) [a]	9.0 (2.8;31.2) [a]	0.19	0.2 (0.1;8.4)
TGF-β1 (ng/ml)					
2 mo	59.8 (52.7;65.0)	60.3 (53.7;65.3)	58.5 (48.2;67.4)	0.63	55.3 (50.8;66.7)
4 mo	57.2 (50.;67.6)	59.3 (53.1;72.1)	61.3 (56.1;69.1)	0.13	57.2 (51.5;62.1)
6 mo	54.7 (47.5-61.6)	57.7 (49.5;67.2)	57.3 (52.1;65.7)	0.13	52.3 (46.0;62.2)
TGF-β2 (ng/ml)					
2 mo	1.2 (1.0;1.4)	1.2 (1.0;1.4)	1.2 (1.0;1.3)	0.13	1.1 (1.0;1.3)
4 mo	1.3 (1.1;1.7)	1.3 (1.1;1.8)	1.4 (1.1;1.8)	0.70	1.2 (1.1;1.5)
6 mo	1.3 (1.1;1.6)	1.3 (1.1;1.8)	1.3 (1.0;1.5)	0.91	1.2 (1.0;1.4)

SF = standard formula. α-lac-EW = experimental formula with α-lactalbumin-enriched whey. CGMP-RW = experimental formula with reduced CGMP whey. TNF = tumor necrosis factor, IL = interleukin, TGF = transforming growth factor. Data presented as median (25th; 75th percentiles). P-values; differences between FF groups using Kruskal Wallis test, post-hoc Bonferroni. [a] Significantly different vs. BF.

Discussion

Narrowing the gap between FF and BF infants regarding growth, metabolic status and health parameters during infancy is a main target, which may also have positive effects in childhood to reduce the risk of later overweight and obesity.

This research project investigated the effects of feeding infant formula with reduced protein content but with enhanced protein quality compared to feeding standard infant formula or breast milk during and after the intervention.

The main finding of this study was that growth was mostly higher in all FF groups compared to the BF group during intervention, but more comparable in the low-protein formula groups compared to the BF infants during the post-intervention period, than if SF had been given. Combined with findings of more similar serum insulin in the low-protein groups and the BF group at 12 months, this may indicate that feeding low-protein formula during early infancy, probably through reduced serum BCAAs, could induce longer term effects on growth and metabolic status. However, despite modifications of formula protein concentration and composition, the metabolome in serum, urine and faeces still differ between formula-fed and breastfed infants. Furthermore, the lack of effects on the faecal metabolome of an increased concentration of α-lac in infant formula may also indicate that other factors than protein affect the gut microbiota and thus the immune response.

Infant growth during intervention

Despite the reduced protein concentration to 1.75 g/100 kcal in the modified infant formula, slightly below the EU regulation level of 1.8 g/100 kcal, higher gains in length and weight were still found in all FF groups during intervention compared to BF infants, except between 4 and 6 months where weight gain did not differ between the α-lac-EW and BF groups. There were no differences in growth parameters between formula groups, which is in contrast to previous studies where higher weight gain was reported in infants fed standard infant formula with a higher protein level than in those fed formula with lower protein concentration (51, 54, 56, 122).

The lack of differences in growth rates during the intervention between the SF and the low-protein formula groups in this study is most probably caused by small difference in protein concentration between SF and the low-protein formulas, which

has also been reported by others (73, 111). However, our findings of more similar BMI at 6 months, and similar weight gain from 4 to 6 months in the α-lac-EW and the BF groups, may result from an improved protein composition, more similar to breast milk.

The findings of no differences in length gain or of head circumference among SF and the low-protein formula groups show that the lower protein concentration in the low-protein formulas is sufficient to support normal growth.

Nutrient intake

Our findings of higher intake of infant formula in the SF group compared to both low-protein groups during most of the intervention period could be due to the higher potential renal solute load (PRSL, i.e. sum of dietary nitrogen, sodium, chloride and potassium (123) in SF infants, caused by higher protein level in SF. This may have caused increased thirst and thus a higher formula intake to maintain hydration. In fact, the analyses of urine metabolomics indicated higher effective urine osmolarity in FF than in BF infants. Furthermore, lower urine urea concentration and aligned urinary osmolarity were found at 4 months in infants fed low-protein formula compared to SF infants. Very high protein overload from formula has previously been shown to result in kidney hypertrophy, at least temporarily (124). Reducing protein content in infant formula may thus reduce the stress on the kidneys.

The higher intake of formula in the SF group resulted in an increased energy intake. The lower intake of infant formula in both low-protein groups, resulting in lower energy intakes, despite isocaloric formulas, could indicate a higher satiety among infants fed low-protein infant formula with α-lac-enriched whey or CGMP-reduced whey, resulting in a further reduced protein intake. Regulation of food intake and energy metabolism is complex. Leptin is a satiety-regulating hormone and higher concentrations in FF than in BF have been shown (125, 126). However, serum leptin was similar in all study groups during intervention, consistent with previous findings (97, 127), suggesting that the effects of leptin on growth may by mediated by factors other than protein intake.

Serum insulin, C-peptide and IGF-1 during intervention

Despite lower protein concentration in the two low-protein formulas than in SF, we did not find any significant impact on insulin or C-peptide concentrations during the intervention period. Infants in all formula groups had significantly higher concentrations of these metabolic markers than BF infants, findings which are

consistent with previous studies (72, 97, 122, 127-130). This may be explained by the rather moderate difference in protein concentration between the SF and the two low-protein infant formulas.

IGF-1 is an important growth factor during infancy and is influenced by protein intake. In the CHOP study, high protein intake from infant formula resulted in higher serum IGF-1 concentration, compared to if infant formula with lower protein content or breast milk had been given and was associated with weight gain until 6 months of age, and to mean weight up to 2 years of age (67). In contrast, in our study serum IGF-1 was similar in all FF groups, most likely related to the more moderate differences in protein concentration between standard and low-protein formulas used in this study, in accordance with previous clinical trials with low-protein infant formula (122, 127). The varying results between studies is probably explained by considerable differences in protein concentrations and previous studies report conflicting results of association between IGF-1 levels and growth in BF infants (131-136).

Protein sufficiency

Since tryptophan is the most limiting amino acid when reducing protein intake, it can also be considered as a marker for sufficient protein supply. The findings of similar or higher tryptophan concentrations in both low-protein formula groups compared to BF infants, indicate that study formulas with a protein level of 1.75 g/ 100 kcal provide adequate amount of protein.

Protein metabolism during intervention

In this study, evaluation of protein metabolism was done both with conventional serum analyses of BUN and amino acids, and with metabolomic analyses both in serum, urine and faeces.

Blood urea nitrogen and amino acids

The reduced protein intake in the low-protein formula groups resulted in lower BUN than in the SF group, and more comparable concentrations as in BF infants. The lower BUN could also indicate an improved protein quality when formulas are enriched with α-lac, in accordance with others (107, 108), or when enriched with CGMP-reduced whey. A reduction of formula protein during early infancy has also been shown to lower serum BCAA to concentrations closer to those in BF infants

also in previous studies (53, 55, 66, 67). The lower leucine concentration in the α-lac-EW formula compared to the other study formulas, resulted in similar leucine concentrations in infants fed α-lac-EW formula and breast milk, in consistency with previous findings (108), and indicate an improved protein quality of the α-lac-enriched formula. In the present study, total serum BCAAs (leucine, isoleucine, and valine) in low-protein formula groups were lower than in the SF group, but still higher than in BF infants, which could indicate that protein concentration could probably be further reduced if combined with high quality formula protein.

An intake of protein above physiological needs could lead to persistently higher BCAA levels. Since BCAAs activate the mTORC1 signalling pathways, this chronic activation could induce enhanced skeletal muscle protein synthesis and the development of adiposity and hence accelerate growth (137).

Metabolomics

By analysing metabolomics in the present study, we have achieved a deeper understanding of the effects of different quantity and quality of protein intake during the first half of infancy. Previous studies have reported differences in the serum and faeces metabolome between FF and BF infants (115, 116, 118, 138, 139). The finding in this study of significant differences between BF and FF infants in all three metabolomes in the same study has not been presented previously. An interesting finding is that increased complementary food intake diminished differences seen in the serum and urine metabolomes between the FF and BF infants, in line with previous reports (115), and demonstrate that the metabolome reflects recent dietary intake.

Enhanced protein quality by increased α-lac concentration not only resulted in reduced intake, but also reduced circulating levels of leucine in serum and of its catabolites in serum and urine. Since leucine is an important activator of mTORC1 complex, reducing the protein level and increasing the α-lac concentration in infant formula could lower the risk for overstimulation of mTORC1 signalling pathways, and hence the risk for accelerated weight gain, and eventually reduce the risk of later overweight and obesity.

Amino acids not used for protein synthesis need to be oxidized and execrated to maintain homeostasis in the body. Even though protein intake in the BF group was lower than in all FF groups, BF infants actually had higher ratio of BCKAs/BCAAs, indicating a more efficient BCAA oxidation in the BF group. The apparently lower BCAA oxidation capacity in FF infants lead to increased circulating BCAA and BCKA levels, with the possible enhancement of the mTORC1 activation. Further, BF infants had a more effective disposal of excessive essential amino acids in the urine compared to FF infants. Thus, less efficient BCAA oxidation capacity and less effective disposal of excessive of essential amino acids in FF infants could

contribute to the different growth patterns seen in FF and BF infants. Why these metabolic processes differ between FF and BF infant are not fully understood. Breast milk contains numerous components that are not present in infant formula. For example, high concentrations of human milk oligosaccharides (HMOs) in breast milk (not present in infant formula) have been found to be negatively associated with infant weight and development of adiposity during the first year of life (140, 141). The protective effect of HMOs may be related to its ability to promote a favourable gut microbiota (142).

The metabolites from the essential amino acid catabolism, particularly from BCAAs, were found to be more concentrated in urine than in serum, indicating that analysis of urine could be used as a better source for evaluating essential amino acid catabolism.

Infant growth, insulin and IGF-1 post-intervention

To evaluate if a reduced protein intake from infant formula might lower the risk for later childhood overweight and obesity, short-, and long-term follow-ups are needed. That a very high intake of protein from formula during infancy can increase the risk of enhanced growth and later overweight and obesity has been shown from the CHOP study (56). Follow-up of the CHOP study population at 6 years of age (70), as well as 11 years post-intervention (69) showed persistently higher BMI in those infants fed very high protein formulas during infancy. In contrast, if feeding an infant formula with a moderately elevated protein concentration (2.2 g protein/100 kcal vs. 1.89 g protein/100 kcal) (110), previously FF infants and BF infants had comparable anthropometric outcomes at 4 years (75) and at 7 years of age (74).

In our study, infants fed the low-protein formulas had similar weight gain between 6 and 12 months and more similar BMI at 12 months as BF infants, whereas infants in the SF group had higher weight gains than low-protein formula groups, indicating an influence of early protein intake on growth later in life. Furthermore, the more similar serum insulin and C-peptide concentrations in the low-protein formula groups and BF infants, as well as the positive association between serum insulin at 12 months and weight gain, could indicate that the lower protein intake may have had imprinting effects during early infancy on insulin secretion and insulin resistance later in life. Our results are in contrast to the EPOCH study, where serum insulin concentrations were similar in all FF groups and BF infants 5 months after end of intervention (122). BCAAs in serum influence insulin release, and elevated serum BCAAs have been hypothesised to precede insulin resistance, and to be associated with later overweight and obesity (143-145). That insulin concentrations may impact long-term growth has been shown in a recent study (146), where

elevated insulin levels at 6 years were associated with enhanced growth from infancy until 6 years of age, which could result from increased insulin resistance. A higher body fat mass was suggested as a possible mechanism behind the higher insulin concentration.

Previous studies have investigated insulin and its association to growth after intervention with low protein formulas. Kouwenhoven et al (127) found no association of insulin at 4 months of age and post-intervention growth up to 2 years of age, nor were insulin concentrations at 4 months associated with growth at 7 years of age in the BeMIM study (74). However, in these studies, insulin concentration was only measured at the end of the intervention and not at follow-up, as was done in the present study, thus eventual imprinting effects might not have been detected.

The similar serum IGF-1 concentration in all study groups at follow-up in our study is in contrast to a previous report where IGF-1 was higher in FF than in BF infants five months after intervention (122). Similar to our findings, IGF-1 has been positively associated with mean weight (147, 148) and to BMI (149) during the first year of life. There are conflicting results, with no association between IGF-1 and growth during the first years of life in two studies (76, 129), contrasting to associations between IGF-1 and gains in length, but not in weight during infancy in another study (149).

Our findings of similar growth at follow-up to BF infants in the low-protein formula groups compared to SF group, as well as the lower insulin levels, possibly due to lower BCAA concentrations during the intervention period, could support the early protein hypothesis. Future follow-up studies are needed to conclude whether these findings persist later in childhood.

Iron status

We did not observe any impact of an increased α-lac concentration on hemoglobin, or iron status parameters despite a suggested enhanced absorptive effect of α-lac of iron. Previous research has shown that iron absorption in FF is associated with present iron status (96). In this study, all formulas had an iron concentration of 8 mg/L (current European recommendation is 4–8 mg/L (92), and our results may indicate, in line with the theory of Szymlek-Gay et al (96), that FF infants with sufficient supply of iron from infant formula regulate the iron absorption according to their current iron status. A recent study also showed that in healthy term well-nourished Swedish infants, iron concentration in infant formula can be safely lowered to 2 mg/ L (150).

Gastrointestinal tolerance

Increasing the α-lac content in infant formula has been reported to improve gastrointestinal tolerance (106, 107, 109). However, in our study all three formulas were well tolerated, with no differences among the FF groups, in line with a previous study where α-lac was added (97). Gastrointestinal tolerance is evaluated differently in different studies. In this study, milder regurgitation was considered to be part of a normal feeding pattern and only excessive regurgitation or incidental vomiting were recorded. Thus, minor differences among formula groups might not have been detected.

Immunological effects

In breast milk, many different bioactive components are present in the right proportions, affecting the immune system and protecting the infant from infections. This complex composition of breast milk is impossible to mimic. An expert group has recently recommended that the safety of bioactive components added to infant formula that influence the immune system should be evaluated accurately before included as an ingredient (151). This study has evaluated an increase in the concentration of α-lac, present in the same concentration as in breast milk and where pre-clinical studies found an impact on infection-related outcomes. In our study we found only minor impact on infection-related morbidity and treatment, and no effect on cytokine profiles.

The lower use of antibiotics in the α-lac-EW than in the CGMP-RW group during the intervention, followed by a higher use in α-lac-EW than in the BF group during follow-up are probably due to differences in the acute otitis media incidence between the groups during these periods, where formula protein composition probably has minor effect. Infants in all FF groups received more antipyretics than BF infants despite similar incidence and prevalence of fever. One reason for this difference may be that antipyretic medication is also used to treat pain or discomfort during an infection even if the infant is not febrile.

Our findings of similar incidences of fever and respiratory tract infections in all study groups are supported by a previous study (97), where infants were fed infant formulas supplemented with α-lac (25%), standard infant formula or breast milk.

There are probably several reasons for the lack of observed effects of an increased α-lac concentration in infant formula on infections and inflammatory response in the present study. The fact that there were no differences in incidences of gastrointestinal-, or other infections, in stool consistency or in gastrointestinal tolerance among the FF groups, may indirectly indicate that increased α-lac

concentration did not alter the gut microbiota to be more like that of BF infants. This assumption is also supported by our findings from the faecal metabolome, where BF infants exhibited metabolic indicators of microbial carbohydrate utilization, while FF infants had more metabolites from protein fermentation. Proteins are easily digested and are thus more or less completely absorbed in the upper gastrointestinal tract. Consequently, only low amounts of peptides and amino acids are available for microbes in the colon.

Breast milk, in contrast to bovine milk, contains HMOs that promote colonization of beneficial bacteria like Bifidobacterium (30, 152), whereas FF infants have a different and more diverse composition of their gut microbiota, rich in Firmicutes and Bacteroidetes (26). Since gut microbiota and the infant immune system are closely related, the different gut microbiota seen in FF and BF infants could be one reason for their difference in the incidence of infections (26, 153, 154).

Cytokines are small proteins that are essential for cell signalling in the immune system and for the regulation of immune and inflammatory responses. Different cytokine profiles, with higher levels of proinflammatory cytokines have been found in FF than in BF infants (155). The reason for these differences could be direct effects of bioactive components in breast milk or by their different gut microbiota.

Addition of bioactive components, other than α-lac, to infant formula have been shown to affect the immune response. The impact of carbohydrate has been shown in clinical studies, where infant formula enriched with HMOs was reported to reduce the incidence of respiratory tract infections and also the use of antibiotics and antipyretics, to incidences more similar to BF infants, compared to if standard infant formula was given (156). This impact is most probably due to the ability of HMOs to affect the microbial composition (157), as well as the cytokine profile (158). Other bioactive components present in breast milk, like milk fat globule membrane (MFGM) have also been shown to lower the risk of infections, such as otitis media (159), gastrointestinal- and respiratory tract infections (160), and fever, as well as to lower the use of antibiotics compared to feeding SF (161). These effects from MFGM could be related to its capability to impact the gut microbiota (116), and the humoral immunity (159). Cytokine profiles in infants fed MFGM supplemented formula have been shown to be more similar to those of BF infants, as compared to infants fed SF (160).

Most previous research on effects of α-lac enriched formula on infection-related outcomes and immune modulation are from animal or in vitro studies (98, 100, 101, 162). Only minor impact on the human gut microbiota has been reported (163). This emphasizes that findings in vitro and from animal studies need to be validated in large human studies. In addition, α-lac-enriched formula has been suggested to protect against infections with Escherichia coli, Shigella flexneri and Salmonella typhimurium (101), but gastrointestinal infections caused by these pathogens are rare in the Swedish infant population. Potential effects of α-lac were thus difficult

to evaluate in our study population, consisting of well-nourished infants living in a privileged setting with high socioeconomic status, with a low burden of infections, a high attendance to the national childhood immunization program, as well as good access to healthcare, all variables contributing to favourable health status in infants. Performing clinical studies in different settings can therefore be valuable.

We analysed a panel of seven cytokines, both with pro-, and anti-inflammatory characteristics to get a broad picture of the inflammatory response. To our knowledge, this has not been done before in infants fed α-lac-enriched infant formula. Our findings of no differences in any of the cytokines between the FF groups or in comparison to BF, except for IL-6, may indicate that no difference exist. However, concentrations change rapidly over time and comparison of levels of individual cytokines to evaluate inflammatory status are thus challenging.

The finding of increased IL-6 concentrations in FF infants during intervention, whereas IL-6 remained at baseline levels in BF infants, also reported in a previous study (164), is interesting and could be related to non-infectious chronic low-grade inflammation. Elevated IL-6 concentrations have previously been associated with overweight in older children (165, 166). Further studies are desirable to investigate the cause of the higher IL-6 concentration during early infancy in FF infants.

In conclusion, our data indicates that most likely other bioactive components than α-lac influence immune physiology to be more similar to breastfed infants, but with the caveat that the outcomes in the present study were only evaluated in a healthy study population.

Strengths and limitations of the ALFoNS study

A strength of this study is the RCT design with a large number of participants and a low drop-out rate, both during the intervention and at follow-up. Since most infants received only low amounts of complementary feeding during intervention this only marginally influenced our ability to evaluate the effects of different formula protein during intervention. Another strength is its design with analyses of markers of protein metabolism, of other metabolic markers and of cytokines both at baseline and then repeatedly during intervention and some also post-intervention, making it possible to evaluate the impact of the intervention and at follow-up. The analyses of all three metabolomes (serum, urine and faeces) in a clinical study on infants is unique and a strength of this study and has to our knowledge not been reported previously.

A limitation of this study is that body composition was not measured. Body composition assessment would have added information about potential impact from the intervention on distribution of fat-mass and fat-free mass in FF infants. BMI is

an indirect measurement of adiposity and weight-for-length data can be used for evaluation of body proportionality (167).

Another limitation is that the consumed volume of breast milk was not measured, only when the infant was breastfed during the registration periods. We considered this measurement to be a burden to the families that might jeopardize their participation.

Possible factors impacting the results

A factor that might have influenced our results was that infants were not enrolled until 5-8 weeks of age (mean age 7 weeks), whereas other studies have included infants earlier (56, 106, 108, 109, 168). The reason for not including infants earlier was that we did not want to interfere with the establishment of breastfeeding. Since 80 % of the FF infants in this study had received breast milk to some extent before being included in the study, and since it is known that early nutrition influences the establishment of the gut microbiota (169) this might have impacted our results related to immune response and infection-related morbidity.

Another aspect that is suggested to contribute to different growth patterns in BF and FF infants, and that was not evaluated in this study, is related to the actual feeding mode, breast or bottle, and the interaction between the infant and the mother/parent. A responsive feeding practice, where the mother/parent is receptive to infant signals of hunger and satiety and then moderates feeding frequency and volumes, has been shown to influence normal growth during the first 2 years of life (170). Parents that bottle-feed are able to control the dietary intake in another way than parents of BF infants. Bottle-fed infants may thus develop less self-regulation of dietary intake than BF infants (171), that might contribute to the higher weight gain seen in FF infants (172). However, in a Swedish study, parents of FF infants were reported to have the same level of parental feeding control as parents of BF infants. Factors like high socioeconomic status and awareness and sufficient support from healthcare providers regarding different feeding modes related to growth outcomes were pointed out as important (173). The infant-parent interaction in the feeding process is complex and many different factors contribute to the level of parental control. However, a high weight gain during early infancy may induce stress in the parents that leads to an increased parental controlled feeding regime regardless if formula or breast milk was given (170, 173). In our study we did not collect information from parents regarding their beliefs, attitudes or practice of feeding.

The purpose of doing research to further improve infant formula is to achieve outcomes more similar to BF infants, and therefore comparisons to BF infants are needed. In this study, as in many other infant formula studies, the BF group was not randomized. To randomize an infant to be BF or to be FF would be unethical and

not possible. In present study, the same inclusion and exclusion criteria were used for both FF and BF infants and FF and BF infants were included in a three to one pattern thought the inclusion period.

Ethical aspects of this research project

Are there any ethical issues when doing research trying to improve infant formula?

There is no doubt that breast milk is the best source of nutrition for the infant, and efforts to increase breastfeeding rates are of high priority. There are various reasons why not all infants are breastfed, such as low access to healthcare providers that can support breastfeeding, socioeconomic circumstances, differences in national regulation of maternity leave and sometimes medical conditions or medication of the mother. Additionally, many families today choose to mix breastfeeding and formula feeding. In order to reach the WHO goal of at least 70% of the world's infants being exclusively breastfed until 6 months of age by 2030, improvement strategies are required at many levels. The healthcare system must provide parents with qualified breastfeeding counselling and support, but also changes in social policy with better conditions for parental leave and working conditions that enable continued breastfeeding are needed (174). However, many infants world-wide are dependent on infant formula and infants cannot decide for themselves what type of nutrition they should receive. Consequently, we must continue to improve infant formula composition by further research to be able to offer all infants the best possible early nutrition.

For obvious reasons, infants cannot give their informed consent to participate in a clinical study. When conducting research in a vulnerable population, we have to be extra cautious. In this study blood sampling was done at some study visits, that might be painful and cause stress for the infant. To minimize the potential discomfort for the infant, anaesthetic creme was use prior to sampling. Furthermore, our study nurses had long experience in taking blood punctures, as well as working with infants and families, and were skilled to notice if any situation became too stressful to the infant or the parent. The low drop-out rates from the study indicate that the procedures at the study visits and at home, with documentation by dietary and symptom diaries, were experienced as meaningful and not stressful for the families.

Another ethical aspect is that this research was supported financially by an industrial company. However, to conduct randomized controlled trials with large numbers of participants are costly and, in this study, important extensive biochemical analyses such as metabolomics were expensive. To attain financial support from others to improve infant formula is difficult. Thus, most studies are dependent on industrial support. However, our research group owns all data collected in this study, and the

funder had no impact on the interpretation of data, nor any impact on what results that should be published. Attention has been drawn to how the marketing of infant formula affects families' decisions to breastfeed or to give infant formula. In the International Code of Marketing of Breast-milk Substitutes from WHO (175, 176), it is clearly stated that breast milk is the most optimal nutrition for infants and that breastfeeding should be promoted and protected from aggressive marketing of infant formula and adherence to this codex must be strict.

Conclusions

- Feeding low-protein formula with 1.75 g protein/100 kcal, slightly below the EU regulation of 1,8 g protein/100 kcal, with either α-lac-EW or CGMP-RW, during early infancy, proved to be safe and resulted in normal growth, still slightly higher than in BF infants (Paper I).
- Feeding low-protein formula with either α-lac-EW or CGMP-RW, during early infancy, resulted in similar BUN as in BF infants, but still slightly higher BCAAs, even though lower than if SF had been given (Paper I).
- Formula protein concentration can probably be further reduced if protein quality is kept high, since growth remained slightly higher in the low-protein formula groups compared to BF group, as did indices of protein metabolism (Paper I).
- Similar weight gains post-intervention and comparable BMI, insulin and C-peptide concentrations at 12 months in low-protein formula groups and BF infants, could indicate that a reduced protein intake early in infancy, through lower BCAAs, could influence growth post-intervention through a lower insulin secretion (Paper II).
- Reduction of protein quantity and improvement of protein quality in infant formula resulted in a serum metabolome more comparable to breastfed infants (Paper III).
- Despite modifications of formula protein quantity and quality, BF infants eliminated excessive amino acids and their side-products more efficiently than FF infants. Thus, protein adjustment of formula alone does not fully account for metabolic differences between FF and BF infants (Paper III).
- The lack of specific effect of increased α-lac concentration in formula on inflammatory response or morbidity, as well as the different faecal metabolome in BF than in all FF groups throughout the intervention, indicate that other factors than protein could influence the gut microbiota and thus probably the immune response (Paper III, IV).

Future perspectives

Despite findings from this research project and from previous research, many questions still don't have a clear answer and future research to try to optimize the composition of infant formula is needed.

Since growth was still slightly higher in the low-protein formula groups than in BF infants during the intervention, RCT´s evaluating safety and efficacy in infant formulas with high protein quality but with even lower protein content than in this study may be possible.

To be able to conclude if the dietary modifications studied in this thesis will prevent childhood overweight and obesity, long-term follow-up is needed. In the ALFoNS study, the study population will be followed until preschool age and forthcoming results will show if any persistent effects on growth and metabolic status, such differences in insulin resistance, have been achieved.

We have learned from the metabolomic analyses that the different metabolic phenotype seen in FF and BF infants are probably not only related to differences in protein intake. Future studies are important for better understanding of the differences between the effects of breastfeeding and formula-feeding on growth and metabolism.

The finding that amino acid catabolic products, especially from BCAAs, are more concentrated in urine than in serum could be helpful in forthcoming studies that intend to evaluate the metabolic response of dietary proteins. Additionally, to collect urine samples from infants is easier than to collect blood samples.

Besides focusing on growth outcomes and metabolic health, an important goal for optimizing infant formula composition is to impact the immune response and infection-related morbidity to improve health outcomes in FF infants.

Populärvetenskaplig sammanfattning

Bakgrund

Bröstmjölk (BM) är den bästa näring för det snabbt växande spädbarnet då den innehåller alla nödvändiga näringsämnen i rätt proportioner. Ammade barn har lägre risk för infektioner i övre luftvägar, öron och magtarmkanal, liksom för övervikt och fetma i barndomen och diabetes typ 2 i vuxen ålder. Om spädbarnet inte ammas är en modersmjölksersättning (MME) det enda alternativet näringsmässigt.

Även om MME utvecklats genom åren för att likna bröstmjölkens sammansättning, finns det fortfarande stora skillnader mellan ammade barns näringsintag och ämnesomsättning, jämfört med dem som får MME. MME görs från komjölk där proteinkoncentrationen är tre gånger högre än i BM och där proteinets sammansättning skiljer sig radikalt åt. Genom åren har man, baserat på forskningsstudier, successivt kunnat sänka proteinnivån i MME och också försökt anpassa proteinets sammansättning så att den ska bli så lik BM som möjligt. Proteinnivån i MME är dock fortfarande högre än i BM för att man inte ska riskera brist på de essentiella aminosyrorna som kroppen inte kan producera själv. Detta gör att barn som får MME har högre serumkoncentrationer av aminosyror, proteinets minsta beståndsdelar, men också av urea som bildas då proteinet bryts ner. Dessutom har man funnit högre insulinnivåer hos barn som får MME jämfört med ammade barn. De grenade aminosyror (BCAA) har särskilt stor betydelse för tillväxten. Det högre intaget av protein kan vara en bidragande orsak till att barn som får MME har högre tillväxthastighet under tidig barndom, vilket kan leda till ökad risk för övervikt och fetma senare under barndomen.

Alfa-laktalbumin (ALA) är det dominerande vassleproteinet i BM men med låg koncentration i MME. ALA har hög koncentration av essentiella aminosyror. Tack vare nya vassleprodukter kan man nu öka koncentrationen av ALA i MME och därmed sänka proteinnivån MME och närma sig den proteinnivå som finns i BM.

Tarmfloran och utvecklingen av immunförsvaret hos spädbarnet är tätt sammankopplade. Den högre ALA nivån i BM skulle enligt djurstudier kunna vara en del av förklaringen varför ammade barn har lägre förekomst av infektioner då ALA sannolikt kan främja en mer gynnsam tarmflora.

Det går inte att helt efterlikna bröstmjölkens unika sammansättning, men då inte alla barn kan eller får ammas är det viktigt att fortsätta att utveckla MME för att minska

skillnaderna avseende tillväxt, ämnesomsättning och hälsa mellan barn som ammas respektive får MME.

Avsikten med denna avhandling var att utvärdera om en reducerad proteinnivå i MME med tillägg av olika koncentrationer av ALA, skulle ge en tillväxt, ämnesomsättning och hälsa som mer liknar det ammade barnets än om vi ger en standard MME ges, liksom om effekterna i så fall kvarstår under hela första levnadsåret.

Metod

I ALFoNS (ALFa-laktalbumin och Nutrition till Spädbarn) studien, en dubbel-blind, kontrollerad studie randomiserades (lottades) 245 spädbarn som redan åt MME till att få antingen en standard MME eller en av två MME med lägre proteinnivå och med högre (27%) respektive lägre (14%) ALA nivå. Åttio barn som helammades inkluderades som en referensgrupp. Alla barn var frisk, fullgångna (födda mellan graviditetsvecka 37–41) och med normal födelsevikt. Mellan 2 till 6 månaders ålder (interventionsperioden) fick spädbarnen den tilldelade MME, och under denna period ombads familjerna att enbart ge studie MME. Från 4 månaders ålder kunde dock små smaksensationer av tilläggskost ges i enlighet med nationella rekommendationer.

Under studietiden följdes barnens tillväxt regelbundet med kontroll av vikt, längd och huvudomfång och via dagböcker rapporterade familjer om kostintag, välmående/sjuklighet, sömn och tarmfunktion. Blodprov togs och urin-, och avföringsprover samlades in för att kunna utvärdera ämnesomsättningen och den inflammatoriska aktiviteten i kroppen.

I denna studien har vi förutom traditionella blodprovsanalyser, använt oss av en speciell teknik, metabolomik, för att undersöka ämnesomsättningen på ett detaljerat sätt. Metabolomik handlar om att kartlägga de kemiska mönster som cellernas ämnesomsättning lämnar i kroppen, där olika typer av kost skapar olika mönster. Dessa metaboliter kan spåras i blod, urin och avföring vars sammanlagda mönster visar vilken påverkan kosten haft i kroppen.

Resultat

Barnen som fick MME med minskat proteininnehåll hade en normal tillväxt under interventionsperioden (2–6 månader) och något högre tillväxt än de barn som ammades. Under tidsperioden 4–6 månader hade dock barnen i MME gruppen med hög ALA nivå en viktuppgång som var jämförbar med de barn som ammades. Barnen som åt MME med lägre proteinnivå hade en ureanivå, biprodukt från nedbrytning av protein som liknade den hos ammade barn och som var lägre än hos de barn som åt standard MME. Nivån av essentiella aminosyror var lika eller högre hos barn som fick MME med låg proteinnivå i förhållande till barn som ammades. Detta tyder på en ökad proteinkvalitét i MME med lägre proteinnivå. Den totala nivån av BCAA var lägre i MME grupperna med lägre proteinnivå än i gruppen som fick standard MME, men fortsatt något högre än i den ammade gruppen. Nivån av aminosyran leucin var lägre hos barn som fick MME med hög ALA nivå och mer lik den hos ammade barn jämfört med de två övriga MME grupperna. Under interventionsperioden noterades inga skillnader i insulinnivå mellan de tre MME grupperna som alla hade högre insulinkoncentration jämfört med den ammade gruppen.

Metabolomik analyserna visar att uppfödning med bröstmjölk eller MME ger olika kemiska mönster i kroppen. Genom att sänka proteinnivån och förbättra proteinkvalitén i MME blev dock mönstren i blodet mer likt de mönster som sågs hos ammade barn. Alla MME grupper uppvisade en reducerad kapacitet att bryta ned BCAA jämfört med den ammade gruppen och utsöndringen av ”överblivna” aminosyror i urinen var lägre hos alla MME grupper. Det kemiska mönstret i avföringen skiljde sig åt mellan barn som fick MME eller BM.

Efter interventionen fann vi att barn i båda MME grupperna med lägre proteinnivå hade en viktuppgång mellan 6–12 månader och ett BMI vid 12 månader som var jämförbar med barnen i den ammade gruppen, medan barn som hade fått standard MME fortfarande växte snabbare än den ammade gruppen. Även insulinnivån vid 12 månader var mer lik den hos ammade barn bland de som fått MME med lägre proteinnivå än bland dem som fått standard MME.

Förekomsten av infektioner och feber skiljde sig inte åt mellan studiegrupperna och alla barn var förhållandevis friska. Vi såg dock att alla barn som åt MME, fick febernedsättande medicin oftare än barn som ammades. Nivån i blodet av protein som hjälper till att reglera inflammationsaktiviteten i kroppen, cytokiner, skiljde sig inte heller åt mellan studiegrupperna, förutom interleukin-6, som var högre i alla grupper som åt MME jämfört med barn som ammades.

Slutsatser

Denna avhandling visar att barn som får MME med lägre proteinnivå och förbättrad proteinkvalité har en normal tillväxt och ämnesomsättning, vilket visar att den aktuella sänkningen av protein är på en säker nivå. Då tillväxten och BCAA nivåerna i blod var något högre hos barnen i MME grupperna med lägre proteinnivå än hos ammade barn, kan sannolikt proteinnivån i MME sänkas ytterligare så länge proteinkvalitén är hög.

Då tillväxtmönstret efter interventionen liksom insulinnivåerna var jämförbara vid 12 månaders ålder hos barn som fått MME med lägre proteinnivå och ammade barn talar detta för att MME med lägre proteinnivå och ökad proteinkvalité som ges under första levnadshalvåret påverkar kroppen med en minskad insulininsöndring och därmed en långsammare tillväxthastighet, mer lik den hos ammade barn.

Den förbättrade sammansättningen av MME i denna studie medförde att barn som fått denna ersättning hade en ämnesomsättning som mer liknade den hos ammade barn, jämfört med de barn som får traditionell ersättning.

Trots att vi har förändrat koncentrationen och sammansättningen av proteinet i MME finns det fortfarande skillnader i ämnesomsättningen mellan barn som fått MME och ammade barn. Barn som ammas har en fortsatt mer effektiv omsättning av BCAA i blod och en bättre förmåga att göra sig av med de aminosyror kroppen inte behöver. Dessa skillnader kan också bidra till olika tillväxtmönster hos barn som får MME respektive ammas.

En ökning av innehållet av ALA i MME hade ingen effekt på förekomsten av infektioner eller på det inflammatoriska svaret i kroppen. En möjlig orsak kan vara att ALA inte påverkar tarmfloran och därmed inte heller utvecklingen av immunförsvaret, åtminstone inte hos friska barn. Orsaken till skillnaderna i IL-6 nivån mellan barn som får MME eller ammas skulle kunna bero på en låggradig kronisk inflammation hos barn som får MME, vilket får undersökas i framtida studier.

En längre uppföljningstid behövs för att undersöka om de positiva effekter vi har sett på tillväxten under tidig barndom av ett minskat proteinintag under första levnadshalvåret kvarstår och därmed kanske kan minska risken för övervikt/fetma senare under barndomen.

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