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Effects of infant formula composition on long-term metabolic health

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Short Title: **Infant formula and long-term metabolic health**

Abstract

Early nutrition may have long-lasting metabolic impacts in adulthood. Even though breast milk is the gold standard, most infants are at least partly formula-fed. Despite obvious improvements, infant formulas remain perfectible to reduce the gap between breastfed and formula-fed infants. Improvements such as reducing the protein content, modulating the lipid matrix, and adding prebiotics, probiotics and synbiotics, are discussed regarding metabolic health. Numerous questions remain to be answered on how impacting the infant formula composition may modulate the host metabolism and exert long-term benefits. Interactions between early nutrition (composition of human milk and infant formula) and the gut microbiota profile, as well as mechanisms connecting gut microbiota to metabolic health, are highlighted. Gut microbiota stands as a key actor in the nutritional programming but additional well-designed longitudinal human studies are needed.

Key words: early nutrition, gut microbiota, metabolism, neonatal feeding, nutritional programming

Over the last decades, metabolic diseases such as insulin resistance (IR), type-2 diabetes (TD2) and obesity have dramatically increased in both children and adults. Thus, worldwide prevalence of obesity has more than doubled between 1980 and 2014.¹ The pandemic of obesity and associated comorbidities may find part of its origin in the early postnatal life. Indeed, according to the developmental origins of health and disease (DOHaD), the foetal period and the first few months of postnatal life are a critical window for determining the flexibility of the system to cope with challenges in later life.^{2,3,4} Quality and quantity of early nutrition plays a crucial role since it can have a great influence on developing infants' metabolism, impacting weight gain, adiposity and energy metabolism on the short and long-term through physiological and behavioural pathways.⁵ Breast milk is recognized as the ideal nutrition for the full-term newborn. An exclusive breastfeeding for the first six months of life is therefore recommended by the World Health Organization. However, despite these recommendations, breastfeeding rates remain low. In 2013 in the United States, 81.1% of infants were breastfed at birth but only 22.3% were exclusively breastfed at the age of 6 months.⁶ The same occurs in Europe, despite large disparities between countries, with breastfeeding rates beyond 4-6 months well below optimum levels.⁷ When breastfeeding is not possible or wanted, the only alternatives are infant formulas (IF). Despite obvious improvements over the past 50 years, IF remain perfectible to better approach the physiologic effects of breast milk. The objective of this review is to summarize differences between breastfed and formula-fed infants on long-term metabolic health, focusing on vaginal, term-born, healthy infants. It will then highlight the modifications that have already been made and that can further be made to IF in order to better mimic the physiological effects of breast milk on both the short- and long-terms. Finally, it will attempt to approach the mechanisms, focusing on the ones involving gut microbiota, as it is known to be impacted by early nutrition and has a key role in regulating metabolism. For space reasons, the effects of early-life

nutritional exposures on infections and immune diseases such as allergy and the interactions between gut microbiota and the immune system, recently published elsewhere,^{8,9} will not be addressed in this review. Similarly, behavioural mechanisms, such as programming of food preferences and eating behaviour, will not be discussed, even though the early postnatal period is known to be essential for the establishment of odors and taste preferences and their maintenance in later life.^{10,11,12,13}

Metabolic health benefits of breastfeeding compared to formula-feeding

Early nutrition is known to play a fundamental role in regulating body development and maturation of tissue functions with short- and long-term organ- specific and time of intervention-specific responses.^{14,15} The first months of life are thus a critical time for preventing metabolic and cardiovascular disorders and obesity in later life. Infant growth trajectory and weight gain during the first year of life are one of the best predictors of obesity in later life, with an increase of 1 SD in weight z-score being associated with a 2-fold higher obesity risk in childhood and a 23% higher obesity risk in adulthood.^{16,17} More precisely, weight gain during the first 6 months of life is a better indicator of body composition in adolescence than weight gain from 6 months to 2 years of age.^{18,19} The early pattern of body fatness is also indicative of later obesity: obese adults have often had an earlier adiposity rebound (at 3 years of age) than non-obese adults (at 6 years of age).²⁰ Indeed, the early period of life is a sensitive period during which adipose tissue expands dramatically since the proliferative capacity of adipose precursor cells from sub-cutaneous adipose tissue is at its highest.²¹ Therefore, numerous studies aiming at understanding the relationship between nutrition, growth in the first years of life and later risks for cardio-metabolic disorders focused on the comparison between breastfed and formula-fed infants.

Growth and body composition

Breastfed infants have a slower growth trajectory during the first months of life compared to formula-fed infants.^{22,23,24,25} The advanced growth tempo, higher weight gain and earlier adiposity rebound of formula-fed children could lead to a higher obesity risk.^{20,23,26,27,28} Indeed, formula-fed neonates would have a 22% higher risk of obesity in infancy compared to breastfed infants.²⁹ Although the magnitude of breastfeeding protection may decrease over time, a 13% reduction in overweight and obesity has still been observed among adults.³⁰ An exclusive breastfeeding would be more efficient to prevent childhood obesity than mixed-feeding (breast milk and IF), itself more efficient than exclusive formula-feeding.³¹ Introducing an IF before 3 months may indeed increase odds of rapid growth in the first 6 years and is associated with higher mean body mass index (BMI) 20 years later.²⁷ However, the results of other clinical studies do not allow a clear conclusion on the relationship between an exclusive breastfeeding and a lower risk of childhood obesity.^{32,33,34,35} The influence of non-investigated confounding factors such as the composition of human milk and its oligosaccharide content may explain the discrepancies between studies.³⁶ As Beyerlein and von Kries already stated, it appears doubtful whether there will ever be a study conducted that will have the appropriate methodology and the statistical power to conclude for or against a potential protective effect of breastfeeding against childhood overweight.³⁷

Metabolism / Metabolic disorders

Insulin resistance and type-2 diabetes

Milk composition has a strong influence on metabolic programming. Besides, weight gain between birth and 3 years of age predicted insulin sensitivity, BMI and waist circumference at the age 8 years.³⁸ Formula-fed infants are at higher risk for IR and T2D in later life than the breastfed ones.^{30,39,40} Much higher urinary C-peptide concentrations (used as a measure of

insulin secretion) were found in formula-fed infants compared to breastfed infants⁴¹ as well as higher pre- and postprandial blood glucose levels.³⁹ If higher glycaemia of formula-fed compared to breastfed infants no more persist in later life, differences in insulinaemia do continue. Breastfed infants have 3% lower insulin levels in later life (childhood and adulthood) compared to the formula-fed ones.³⁹ At 8 years of age, overweight and obese infants formula-fed for 4 months or longer were more insulin-resistant than the ones that were breastfed, and they compensated their lesser sensitivity by a higher insulin secretion.⁴² These greater insulin levels in formula-fed infants may explain their greater deposition of subcutaneous adipose tissue⁴³ and may be due to a faster increase in serum branched-chain amino acids⁴⁴ as demonstrated in both animal⁴⁵ and human^{46,47,48,49,50} studies.

Cardiovascular risks factors

Early nutrition may also have an impact on cardiometabolic risk factors (dyslipidaemia and hypertension) and atherosclerosis in later life. Formula-fed infants could be at higher risk of atherosclerosis as a negative correlation has been found between inflammatory markers serum levels (serum monocyte chemoattractant protein-1 level and uric acid) and duration of breastfeeding.⁵¹ Besides, observational studies suggested that formula-feeding could be associated with higher blood pressure levels in childhood compared to breastfeeding.^{52,53} A prospective study on Mexican children concluded that an exclusive and prolonged breastfeeding had a beneficial effect on later cardiometabolic health through lower total cholesterol, low-density lipoprotein cholesterol and triglycerides levels at the age of 4.⁵⁴ Some studies demonstrated that these lower levels persisted even later in adulthood (from 17 to 64 years).⁵⁵ Yet, earlier at 4 and 8 weeks of life, formula-fed infants have lower serum cholesterol, triglyceride and transaminase level (ALAT, ASAT, γ GT) compared to breastfed infants.⁵⁶ The higher levels of cholesterol in breastfed infants before 1 year of age can be

explained by the higher level of cholesterol in breast milk compared to IF made with vegetable oils.⁵⁵ The twist in cholesterol levels between formula-fed and breastfed infants may be due to a nutritional programming of cholesterol synthesis by early postnatal diet.⁵⁷ In a pig model, the lower serum cholesterol concentration in formula-fed piglets compared to the breastfed ones was associated with an increase in mRNA encoding cholesterol 7 alpha-hydroxylase.⁵⁸ Despite the above studies, the long-term benefits of breastfeeding on preventing cardiovascular diseases remain controversial^{30,59,60,61} and further studies are required. Clearer results are mandatory to decipher whether reduced cholesterol level in early life has long-term deleterious consequences or not. A modest decrease in cholesterol level in adulthood could lead to a 5% reduction in coronary heart disease incidence.⁵⁷

Improving infant formulas to approach the physiologic effects of breast milk

Breast milk remains the gold standard and the objective is therefore to improve the composition of IF to better approach its physiologic effects. Breast milk has a unique composition that leads to specific metabolic and physiological responses.⁶² Several factors have been suggested to explain the association between formula-feeding, growth, body composition and later risk of obesity and metabolic diseases. Differences in qualitative and quantitative intake in nutrients, hormones and milk bacteria may be involved. Since IF composition has greatly evolved and been improved over the last decades, cautiousness is needed when interpreting results from older cohorts.⁶³

Macronutrients

Proteins

Cow's milk proteins are the unique source of proteins in most IF but have a lower quality compared to breast milk, partly because of differences in their amino acid contents. Over the last decades, the amount of protein per energy content has generally been higher in IF than in human milk (0.9g/100 mL in mature milk, 1.29-1.38g/100 kcal)⁶⁴ to meet infant's protein and amino acid requirements.⁶⁵ Old studies from the 1990s reported that formula-fed infants aged 3-12 months had 10-18% higher energy intakes and 55-80% higher protein supply per kg of body weight than breastfed infants.^{66,67} A high protein intake in early infancy has been associated with an increased growth and higher later adiposity.^{68,69,70,71} A high protein intake during the first year of life thus affected fat distribution in healthy children with an enhanced pre-peritoneal fat (a marker of visceral fat) but not subcutaneous fat tissue accumulation at the age 5 years⁷⁰ and higher fat mass but not fat free mass at the age 6 years⁷¹. According to the early protein hypothesis, the higher protein content in IF could lead to increased circulating concentrations of insulin-releasing amino acids, stimulating the release of insulin and insulin-like growth factor I (IGF-1) and resulting in an accelerated growth, a faster weight gain and a greater adiposity.⁷² Formula-fed infants have higher levels of IGF-1 than breastfed infants at several ages and IGF-1 levels at 7-8 years of age was associated with the history of breastfeeding.^{73,74,75} The early protein hypothesis was supported by a systematic literature review that assessed that a higher protein intake in infancy and early childhood was convincingly associated with increased growth and higher BMI in childhood, the first 2 years of life being likely most sensitive to high protein intake.⁷⁶ However, inconsistent evidence is available on the association beyond infancy and on later childhood overweight or obesity.⁷⁷

An improvement of IF has consisted in decreasing their protein content without altering the plasma amino acid profile. Two approaches has been considered: an increase in the proportion

of α -lactalbumin^{78,79} and, if necessary, the addition of free limiting amino acids.⁸⁰ However, the addition of free amino acids in IF may have long-term metabolic outcomes since they will be absorbed and oxidized more rapidly than protein-bound amino acids.⁸¹

Infants fed a low-protein IF were lighter at the age 2 but had similar height than infants fed a high-protein IF,⁸² and their weight and BMI as well as their metabolism were closer to the ones of BF infants.⁸³ At the age 6, their obesity risk was lower⁸⁴ and at the age 14-16 years, it is expected to be 13% lower.⁸³ Other factors than IGF-1 may also impact growth velocity since decreasing the IF protein content did not impact plasma IGF-1, insulin and C-peptide concentrations during the first year of life and body composition during the first 60 months of life but affected length and head circumference growth in a French randomised controlled trial.⁸⁵ Decreasing the protein content in IF may also decrease the level of metabolic stress⁴⁵ by decreasing plasma levels of insulinogenic amino acids close to those induced by breast milk as well as urinary C-peptide level.⁴¹

An ideal protein content of 1.8 g/100 kcal was therefore established for standard milk protein-based IF⁸⁶ and protein content in IF is now relatively close to the one found in breast milk.⁸⁷ Human milk also contains bioactive proteins such as lactoferrin (present at higher concentration in human milk compared to IF),⁸⁸ hormones and cytokines that may affect growth, body composition and metabolism in later life and explain differences observed between breastfed and formula-fed infants.^{89,90,91,92} The supplementation of IF with bovine lactoferrin may help narrow the gap between breastfed and formula-fed infants.⁹³ However, long-term and mechanistic studies are still missing.

Fat quality and structure

If in the early 20th century the fat matrix of IF was made of cow's milk fat and butterfat, today the fat matrix of most IF is exclusively made of a blend of vegetable oils. The use of

vegetable oils enabled to better mimic the human milk mono- and polyunsaturated fatty acid profiles²¹ but induced major differences in the fat globule and triglycerides structure. Regarding fatty acid profile, IF usually contain more long-chain polyunsaturated fatty acids (LC-PUFAs) of the ω 3 and ω 6 families due to their supplementation. If ω 3 LC-PUFAs have been associated with improved insulin sensitivity, reduced body weight gain and adiposity and counteraction of dyslipidaemia in adult human and animal studies,⁹⁴ data on nutritional programming by postnatal ω 3 LC-PUFAs are limited. In a male murine model, postnatal supplementation with ω 3 PUFAs reduced body fat deposition during adulthood and led to less hypertrophic adipocytes and healthier plasma lipid profile and glucose homeostasis.⁹⁵ Similar beneficial effects were observed with a low ω 6 PUFA diet⁹⁶. Both diets (high in ω 3 and low in ω 6) affected permanently the development of the central regulatory circuits controlling energy balance.⁹⁷ The higher amount of ω 6 fatty acids in IF than in breast milk may promote the adipose tissue development by enhancing the formation of pre-adipocytes²¹ and the arachidonic acid (ARA, ω 6) and its metabolites may directly be involved.^{21,98} However, a recent systematic review has concluded on the lack of evidence on consumption of LC-PUFA-supplemented IF and later risk of obesity.⁹⁹ Moreover, the supplementation of IF with ARA and docosahexaenoic acid (DHA, ω 3) has proven to be efficient to lower blood pressure in children at the age 6 years and potentially their cardiovascular risk in adulthood compared to their counterparts who had received a non-supplemented formula.¹⁰⁰ A balanced ratio between ω 3 and ω 6 is essential^{101,102} however, the addition of DHA (20-50 mg/100 kcal) but not ARA is now mandatory in IF for full-term healthy neonates,¹⁰³ which raises questions regarding the suitability and safety of these IF.^{104,105,106}

Beyond fatty acid composition, the lipid matrix is also of great importance. Indeed, breast milk contains fat globules surrounded by a complex trilayer membrane called milk fat globule membrane (MFGM) rich in phospholipids (~30% of total lipid weight, mainly sphingomyelin,

phosphatidylcholine and phosphatidylethanolamine), cholesterol and proteins (lipid:protein weight ratio of 1:1).¹⁰⁷ Due to homogenization and thermal treatments, the structure of fat in IF is different in size of lipid droplets, interfacial composition and architecture, and fatty acid profile.^{108,109} Besides, IF with a lipid matrix made only with vegetable oils do not contain MFGM. A clinical study displayed no effect of MFGM supplementation in IF on growth and LDL:HDL ratio, but a higher plasma cholesterol trajectory from 2 to 6 months, that did not persist at 12 months of age.¹¹⁰ In another study, providing an IF supplemented with cream and a bovine MFGM concentrate for 2 months normalized cholesterol and LDL concentrations to levels of breastfed infant.¹¹¹ Animal studies can provide a better understanding of the importance of the structure and composition of fat matrix. In a murine model, male pups given an experimental formula with large lipid droplets coated with MFGM mimicking milk fat globules from 16 until 42 days of age displayed lower fat accumulation (by 30%) and lower fasting plasma leptin, resistin, glucose and lipid concentrations as adults compared to mice given a standard vegetable-fat-based formula.¹¹² Their adipocyte size was lower yet not their number and some key regulators of metabolic activity, such as PPAR γ , were less expressed in their white adipose tissue reducing their susceptibility to obesity in later life.¹¹³ Baars *et al.*¹¹⁴ recently demonstrated that both the large droplets and the MFGM coating were mandatory to induce such effects. A suggested mechanism would be a reduction in lipid storage capacity and a decline in lipogenesis in white adipose tissue. In infants, such an IF containing large, phospholipid-coated lipid droplets was found to support adequate growth in healthy Asian infants during the first 4 months of life compared to a standard IF.¹¹⁵

Besides, the supplementation of IF with cholesterol by a direct addition of cholesterol or by replacing a fraction of vegetable oils by dairy lipids may have a beneficial effect on cholesterol level in adulthood. The addition of dairy lipids in IF enabled a normal growth during the 4 months of feeding.¹¹⁶ Supplementation in cholesterol (3.44 mmol/l vs. 0.85

mmol/l in the regular cow's milk protein-based formula) of IF given to full-term healthy neonates did not modify plasma cholesterol concentrations at 4, 11 and 12 months of age. Both cholesterol-supplemented and not-supplemented formula-fed groups differed from the breastfed group at 4 months for plasma total-cholesterol but not at 11 and 12 months.¹¹⁷ In another study, differences were observed at 4 months but did not persist at 18 months.¹¹⁸ The lack of differences might be explained by the short-term follow-ups or by the bioavailability of the added cholesterol (unesterified in formula *vs.* free and esterified in breast milk). The efficiency of cholesterol absorption may also be decreased by phytosterols present in infant formulas, competing in bile salt/lecithin micelles.¹¹⁹ In order to resemble the animal/plant sterol ratio of human milk, plant sterols should be reduced.¹²⁰ Long-term human and animal studies are still needed to conclude on the outcomes of the addition of cholesterol in infant formulas.

Carbohydrates and oligosaccharides

Human milk vs. formula-feeding

Lactose is the main carbohydrate source in both human milk and standard IF and also the most stable of all macronutrients with a concentration of about 67-74.4 g/L (10.3-11.4g/100 kcal) in human milk.^{90,121} Animal studies have demonstrated adverse long-term effects of an increased intake of carbohydrate in early life. Indeed, the supply of a high-carbohydrate (polyose) formula to 4-day-old rat pups during the suckling period led to chronic hyperinsulinemia and adult-onset obesity. These effects were mediated by numerous adaptations in 12-day-old rats targeting pancreatic islets, including the autonomic regulation of insulin secretion, the gut (increased GLP-1 levels) and possibly the hypothalamus. The phenotype was also spontaneously transmitted to the progeny.¹²² Yet, carbohydrates levels in IF do fit that of breast milk and such high carbohydrate IF are not available.

Human milk also contains a high quantity of oligosaccharides (third largest component after lactose and lipids, 5-20 g/L in mature milk) (HMOs), which are unconjugated glycans composed of 5 monosaccharide building blocks (D-glucose, D-galactose, N-acetylglucosamine, L-fucose, and sialic acid derivative N-acetyl-neuraminic acid) associated under more than 200 distinct forms. The total amount and composition of HMOs are highly variable between women, depending on maternal genetic, lactation stages and environmental factors (such as geographic localization, diet and physical activity).^{123,124,125,126,127} Resistant to digestion, HMOs reach intact the distal small intestine and colon where they are fermented by the gut microbiota, sometimes in a strain-specific manner. For instance, *B. infantis* grows well on several HMOs, but most bifidobacterial species only metabolize the lacto-N-tetraose, one of the predominant HMOs.^{123,124,125,126,128} Members of the genus *Bacteroides* are also known to consume specific HMOs.¹²⁹ Different HMOs may therefore differently shape gut microbiota composition and activity through modulation of human milk microbiota.¹³⁰ For instance, 2'-fucosyllactose (2'FL) has been linked to a greater abundance of gut bifidobacteria and infants whose mother are non-secretor would have a delayed establishment of bifidobacteria-laden microbiota,¹³¹ with lasting consequences on the gut microbiota at 2 to 3 years of age.¹³² Milk HMOs can be positively or negatively correlated with a number of bacteria in the stool of breastfed infants.¹³³ In addition to their action on gut microbiota, HMOs may exert direct effects on intestinal epithelial cells^{134,135,136} or potential systemic effects by reaching the circulation.^{137,138} Therefore, either directly or indirectly, they can impact health on both the short- and long-terms. For instance, milk HMO diversity and evenness at 1 and 6 months of lactation were associated to the suckling infant's weight and body composition (lean and fat mass). These preliminary results need to be corroborated by higher sample sizes and longer follow-up to elucidate the exact contribution of specific HMOs to infant development.¹³⁹

Prebiotics in infant formulas

Until recently, individual HMOs were not available at large-scale and therefore could not be added to IF. Furthermore, the complex mixture of HMOs cannot be reproduced in IF. The addition of prebiotics, substrates “selectively utilized by host microorganisms conferring a health benefit”,¹⁴⁰ has been a first step to approach HMOs benefits in IF. Studied prebiotics in IF have mainly been a 9:1 mixture of short-chain galacto-oligosaccharides (scGOS) and long-chain fructo-oligosaccharides (lcFOS), or (used single or combined) GOS, FOS, acidic oligosaccharides, oligofructose, inulin, polydextrose or lactulose, with considerable variations in doses and duration of administration between clinical studies.¹⁴¹ If prebiotic supplementation has been associated with a slightly greater weight gain in a systematic review of randomized controlled trials,¹⁴² small sample sizes and the lack of statistical power and long-term follow-up complicate the interpretation of the effects of prebiotic-supplemented IF. A routine use of prebiotic-supplemented IF is therefore not legally recommended.¹⁴¹ Besides, prebiotics commonly added to IF are much simpler structures than HMOs and cannot reproduce all their benefits, most of them being structure-specific. For instance, prebiotics such as FOS and GOS are known to be broadly bifidogenic whereas HMOs are metabolized by a smaller array of bifidobacteria.¹²⁶ Short-chain fatty acids (SCFAs), such as acetate, propionate and butyrate, are a major product of gut microbiota fermentation. They are a source of metabolizable energy and they can be used as signalling molecules and involved in *de novo* lipid synthesis.¹⁴³ SCFAs may have a beneficial role on weight gain and adiposity in infants.¹⁴⁴ However, prebiotic supplementation does not seem to affect the biochemical parameters in the blood and urine samples¹⁴⁵ and different combination of prebiotics have demonstrated similar efficacy on gut microbiota composition¹⁴⁶ and on growth rate.¹⁴⁷ Long-term health outcomes of neonatal prebiotic supplementation in IF have not been extensively

described yet. A small explorative study on a specific population of hepatitis C virus-infected mothers demonstrated differences in gut microbiota composition at the age of 12 months between infants fed a scGOS/lcFOS-supplemented IF during the first 6 months of life compared with the placebo group.¹⁴⁸

Other potential prebiotics are oligosaccharides present in the milk of farm animals such as cows and goats. Even if their oligosaccharide concentrations are 100-1000-fold lower than in human milk and less diverse,¹²³ they may represent an alternative source of prebiotics.¹⁴⁹ Individual HMOs like 2'FL and lacto-N-neotetraose (LNnT), which account for ~37% of total HMOs, are now commercially available and provide more promising opportunities for the development of IF closer to breast milk.¹⁵⁰ The supplementation with 2 HMOs (2'FL and LNnT) for 6 months did not induce differences in weight, length, BMI or corresponding z-scores through 12 months compared to a non-supplemented IF,¹⁵¹ but induced a faecal microbiota and metabolic signature closer to that of breastfed infants at 3 months of age.¹⁵² No significant differences were further observed for weight, length, or head circumference growth during a 4-month study period between infants fed IF containing GOS and supplemented or not with 2'FL.¹⁵³

If the smallest and most abundant HMOs are now available, the more complex ones are not. Besides, several questions remain regarding which HMO composition should be considered as ideal and therefore which HMOs should be added to IF, their dosage and their short- and long-term health consequences.¹⁵⁴

Probiotics and synbiotics

Formerly considered to be sterile, human milk has recently been recognized as a continuous source of viable commensal and potentially probiotic bacteria such as *Staphylococcus*, *Streptococcus*, *Bifidobacterium* and *Lactobacillus*.¹²⁵ If over 200 different

bacterial species (from 50 different genera) have previously been identified in human milk,¹⁵⁵ recent studies revealed a larger microbial diversity with over 200 different genera and 700 species.^{156,157} The origin of bacteria present in breast milk is not fully elucidated but the current hypothesis is that bacteria from the maternal gut may be trapped by dendritic cells and spread to the mammary gland *via* the lymphatic and blood circulation,¹²⁵ although an additional retro-contamination of mammary gland by infant oral microbiota cannot be ruled out. Core milk microbiomes composed of genera present in most human milk samples have been identified with 9 genera accounting for half of the microbial community¹⁵⁵ or 12 genera accounting for more than 81% and 73% of the taxa identified before week 6 and at week 12 of lactation, respectively,¹⁵⁷ with 3 genera shared between these two studies. Human milk microbiome is affected by external factors such as maternal nutrition, gestational age, health status and delivery mode, and also varies across lactation.^{125,158} The daily ingestion of bacterial cells by an infant receiving 800 mL of breast milk would be up to 10^7 - 10^8 .¹⁵⁹ Therefore in a recent 12-month longitudinal study,¹⁶⁰ it was estimated that breast milk accounted for almost one-third of total bacteria present in the gut of breastfed infants during the first month of life. Moreover, metagenome predictions indicated that breast milk harbours bacteria with prominent carbohydrate, amino acid, and energy metabolism functions, suggesting a dual regulatory role of human milk bacteria as a continuous inoculum and on the physiological and metabolic development of neonates *via* their metabolites.

Introducing bacteria in IF can be a clever way to better adjust IF to breast milk. Such bacteria, called probiotics, are therefore added in IF.

Probiotics

Probiotics are defined as “live microorganisms that, when administered in adequate amounts, confer a health benefit on the host”.^{161,162} The most commonly probiotics used in infant nutrition are strains of *Bifidobacterium* (*Bifidobacterium infantis*, *Bifidobacterium lactis*, *Bifidobacterium longum*) and *Lactobacillus* (*Lactobacillus acidophilus*, *Lactobacillus fermentum*, *Lactobacillus reuteri*, *Lactobacillus rhamnosus*) isolated from the human gastrointestinal tract, human breast milk or dairy products.^{141,163} The effects of probiotics such as the normalization of perturbed microbiota or the production of SCFAs are widespread among probiotics while particular changes in the microbial composition of the gut are specie- or even strain-dependent.^{162,163} Probiotics are usually administered alone or in 2-3 strains combination, far from the microbial diversity of human milk. The efficacy of specific probiotics has been demonstrated for the treatment of acute gastroenteritis and the prevention of necrotizing enterocolitis, antibiotic-associated diarrhoea and nosocomial diarrhoea in infants and children.^{164,165,166}

The long-term effects of early probiotic consumption on growth have been investigated in a few studies 2 to 13 years after the intervention. No effect of supplementation with *L. rhamnosus GG* or *L. fermentum CECT5716* in the first months of life was observed on growth and microbiota composition at 2,¹⁶⁷ 3,¹⁶⁸ 5¹⁶⁹ and 13 years.¹⁷⁰ In contrast, an impact of maternal probiotic supplementation with *L. rhamnosus GG* was observed on children’s body weight development, with a lesser weight gain until 2-4 years, especially among children who later became overweight, resulting in a lower BMI until 7 years of age.¹⁷¹ However, there is no study investigating the effects of probiotic supplementation on metabolic syndrome progression in adulthood. In animal studies, early probiotic administration has been associated with the maintenance of eubiosis, intestinal tract maturation, and improved immunity and reduced pathogen infection.¹⁷²

The ESPGHAN Committee on Nutrition concluded in 2011 that the administration of currently evaluated probiotic-supplemented formula to healthy infants did not raise safety concerns with regard to growth and adverse effects. However, because of high variability in responses, probably due to the small size and insufficient statistical power of studies, the different probiotic strains, doses, timing and duration of administration used, the lack of data on the long-term after the cessation of the probiotic and the different methods used for microbiota analysis, the relevance of supplementation of IF with probiotics remains unclear and the routine use of probiotic-supplemented IF is not currently mandatory.^{141,158,163}

Synbiotics

Supplementation of IF with prebiotics or probiotics alone does not fully mimic the complexity of human milk, which provides both, and has some limitations. Probiotics would transiently colonize the infant's gut¹⁷³ (further studies are needed¹⁷⁴) and prebiotics can only have an impact on bacteria that are already present in the gut. Therefore, beneficial synergistic effects may be expected from a combination of probiotics and prebiotics, called synbiotic, using prebiotics to selectively increase abundance of both endogenous beneficial bacteria and probiotics in the infant gut. However, due to the limited available data on synbiotics, the ESPGHAN Committee on Nutrition does not currently recommend the use of IF supplemented with synbiotics even though the available data suggest that they are safe.¹⁴¹ Besides, the superiority of synbiotics over probiotics or prebiotics is not yet clearly established.¹⁷⁵ The impact on microbial profile, particularly the bifidogenic effect, of synbiotic IF containing *Lactobacillus reuteri* and GOS/FOS, *Lactobacillus paracasei* and GOS/FOS or *Bifidobacterium animalis* and bovine milk-derived oligosaccharides did not

result in any differences in infant growth parameters during the first year of life compared to control IF (without prebiotics and/or probiotics).^{176,177,149}

To conclude, even if some prebiotics and/or specific probiotic strains display promising results, more randomized controlled clinical trials with longer follow-up are needed for the determination of tailored combinations and of their physiological and metabolic impact on the host (Fig. 1).

Effects of the infant formula composition on gut microbiota: a possible mechanistic link?

Early establishment of gut microbiota and differences between formula-fed and breastfed infants

Recent advances in sequencing technologies of the microbiota have questioned the *sterile womb paradigm*, suggesting a mother-to-child transfer of commensal bacteria *in utero*.^{178,179,180,181} If there is currently no clear consensus regarding the prenatal life, it is well established that the first months of life are crucial for the establishment of the gut microbiota and host-microbiota symbiosis. Gut microbiota exerts several functions such as facilitating nutrient utilization, synthesizing amino acids and vitamins, educating the naïve immune system and programming the metabolic system in neonates.^{8,182,183} It modulates infant growth and body composition^{184,185,186,187} and plays a crucial role in lifelong health. In mice, an early exposure to antibiotics during the suckling period was associated with an increased fat mass and a negative modulation of hepatic metabolism in adulthood though gut microbiota was only transiently altered.¹⁸⁸ Mainly consisting of facultative anaerobes and then obligate anaerobes within the 2 first weeks of life, gut microbiota slowly achieves a more complex structure and evolves towards an adult-like configuration throughout the first three years of

life.^{189,190,191} Among the influencing factors of gut colonization, early nutrition plays a predominant role.^{8,192} Formula-feeding has been associated with a less stable microbiota over time, different overall bacterial composition and higher bacterial richness and diversity (although controversial)¹³³ compared to breastfeeding.^{193,194,195,196,197} However, inconsistencies exist between studies on the impact of formula-feeding on gut microbiota composition and may be explained by several factors such as changes in IF composition over time and differences in oligosaccharides composition between breast milk and IF.¹⁶³ Early gut microbiota may influence later microbiota^{198,199} but the sustainability of early microbiota changes in later infancy and adulthood remains uncertain.^{148,167} In addition, alterations in the gut microbiota profile during the first months of life may precede overweight development.^{186,200,201,202} If the exact role of specific bacterial families or genera is not clear, a higher abundance of the genus *Bifidobacterium* has been observed during the first year of life in infants who remained normal weight at 7 years compared to children who became overweight.²⁰² Infants not primarily breastfed had higher abundance of *Bacteroidaceae*¹⁶⁰ and positive correlations were found between a higher abundance of *Bacteroides* spp. (in particular *B. fragilis*) and BMI at the age of 3 and 26 weeks but disappeared at approximately 2.5 years of age.²⁰¹ In overall, these data suggest that gut microbiota primocolonization is crucial and may affect infant growth trajectories. However, further investigations are needed to determine the time length of this early critical window and why early microbiota changes are not always observed in later life.

Beyond the taxonomic level, it is necessary to investigate the infant gut microbiota in terms of functionality since bacteria belonging to different taxonomic groups may perform similar functions.²⁰³ Formula-fed infants had an accelerated functional maturity compared to breastfed infants, characterized by enrichment in functions characteristic of the adult microbiome at 4 months, despite a small overall functional difference.^{204,205} Formula-fed

infants also had high stool levels of SCFAs such as propionate, butyrate, acetate, 5-aminovalerate and free amino acids at 3 and 6 months of age while breastfed infants had high concentrations of fucosylated oligosaccharides and lactic acid, as a result of a higher fermentation of HMOs.²⁰⁶

Gut microbiota can be modulated by the macronutrient composition of IF. A whey-predominant IF led to a faecal microbiota closer to that of breastfed infants at 2 months of age, compared to a casein-predominant IF.²⁰⁷ Proteins, such as lactoferrin, may also function as prebiotics and impact gut microbiota composition.^{208,209} The structure of triglycerides could also affect gut microbiota. Indeed, a high β -palmitate formula was shown to increase *Lactobacillus* and *Bifidobacteria* counts in faecal stools of 6 week-old infants at abundances similar to breastfed infants, compared to infants receiving a low β -palmitate IF.²¹⁰ A recent study in a germ-free mice model demonstrated that the fatty acid composition and phospholipid types may differently affect gut microbiota establishment.²¹¹ The addition of MFGM alone did not lead to changes in gut microbiota composition in 28 day-old piglets compared to standard IF but the addition of both dairy lipids and MFGM affected gut microbiota composition, with an increase in Proteobacteria and a decrease in Firmicutes phyla compared to piglets fed a standard vegetable-oil IF.²¹² These changes were similar to those observed at 3 months of age between breastfed infants and infants fed a vegetable-oil-based IF.²¹³

To conclude, relationships have been described between macronutrients and specific bacteria but further studies are needed to better understand them and evaluate their long-term impact on health.¹⁵⁹

Mechanisms linking gut microbiota and long-term health

Several mechanisms have been proposed to connect gut microbiota to metabolic health. One mechanism relies on the property of gut microbiota to harvest energy in relation with the enrichment of genes coding enzymes that utilize non-digestible dietary carbohydrates to produce SCFAs. Indeed, breast milk butyrate was inversely associated with 12-month skinfolds and BMI, 3-12 month skinfold gain and weight gain in a prospective birth cohort. Associations were also found for acetate and formic acid and BMI and skinfolds at 3 months but not for adiposity at 12 months.²¹⁴ SCFAs are key mediators of the crosstalk between gut microbiota and host cells, able to act as signalling molecules by bonding to their receptors, expressed by different cell types such as enterocytes and intestinal enteroendocrine cells. SCFAs can bind to the free fatty acid receptor (FFAR) 2 and 3 (formerly known as G-protein coupled receptor (GPR) 43 and 41, respectively), the affinity for these receptors depending on the size of their aliphatic tail^{215,216} and stimulate the release of enteroendocrine hormones such as glucagon-like peptide-1 (GLP-1) and peptide YY (PYY). For instance, propionate lowered hepatic glucose production in healthy rats *in vivo*,²¹⁷ and prevented weight gain in overweight adult humans²¹⁸ *via* the stimulation of the release of GLP-1 and PYY.^{219,220} GLP-1 is an incretin exerting several functions, from increasing insulin and decreasing glucagon secretion and stimulating beta-cell growth to decreasing appetite and food intake. GLP-1 secretion is not only stimulated by SCFAs but also by the digestion of macronutrients.^{221,222,223,224}

Bacteria and their metabolites (SCFAs but also other signalling molecules) may also regulate the expression of key regulatory and functional genes such as those that are important for fat storage (adipocyte development and lipolysis) and oxidation, and gastrointestinal hormone production.²²⁵ For instance, butyrate inhibited intestinal cholesterol biosynthesis *in vitro*, thus possibly lowering plasma cholesterol levels²²⁶ and improved insulin sensitivity in mice, through the promotion of energy expenditure and induction of mitochondrial

function.²²⁷ SCFAs also regulate leptin secretion by adipocytes.^{228,229} SCFAs may therefore be mediators of the long-term effects exerted by early nutrition.

Host-microbiota interactions could also be mediated by other mechanisms. Diverse metabolites and signalling molecules produced by gut microbiota such as SCFAs and folate may directly or indirectly modify the epigenome and therefore regulate host genes and shape long-term phenotype.^{230,231} There are three distinct epigenetic mechanisms: DNA methylation, histone modifications and non-coding microRNAs (miRNAs). Epigenome and microbiome would be interrelated and influenced by each other but their interaction in responses to early-life environment remain unclear.^{232,233} SCFAs, such as butyrate and propionate are thought to modulate host cellular processes through inhibition of histone deacetylase activity and to alter the expression of specific genes.^{234,235,236} Interactions between gut microbiota composition and epigenetic regulation of genes have been demonstrated in obese and diabetic adult patients with a significant correlation between a higher BMI and lower methylation of FFAR3.²³⁷ In a germ-free mice model, supplementation with SCFAs was sufficient to recapitulate chromatin modification states and transcriptional responses associated with colonization.²³⁸ Besides, a clear association has been demonstrated in pregnant women between a microbiome dominated by Firmicutes and blood DNA methylation patterns, associated with greater susceptibility to diseases such as cardiovascular disease and obesity.²³⁹ Breastfeeding would contribute, *via* the modulation of gut microbiota composition, to the production of larger amounts of folate, inducing DNA methylation marks. On the contrary, the higher protein content of infant IF may lead to a higher amount of protein reaching the colon and therefore to an enrichment in proteolytic bacteria, producing butyrate, at the expense of carbohydrate-fermenting bacteria, promoting histone acetylation, known to be a key factor of epigenetic regulation of cholesterol and lipid metabolism.²⁴⁰ Other nutrients such as LC-PUFAs may also have an effect on epigenetic processes.^{241,242} Thus, both the

supplementation of IF with probiotics and prebiotics and its composition in macronutrients may induce epigenetic modifications by impacting gut microbiota composition and metabolism.

Early life nutrition may also alter miRNA profiles and therefore gene expression since miRNA expression depends on gut microbiota community.^{231,243,244} Human and bovine milks contain miRNAs and so do IF but with much lower expression.^{245,246,247,248}

Targeting gut microbiota seems to be relevant to prevent metabolic diseases on the long-term. Integrative approaches of metagenomic, epigenetic and metabolomic/lipidomic data are necessary to better understand their dynamic interactions with early-life environment and improve long-term health (Fig. 2).

Conclusion

Early nutrition plays a predominant role in health and well-being of the newborn and in later life by modulating its metabolism. Improving the functional effects of IF to reduce the gap between breastfed and formula-fed infants is crucial and has been the topic of great research over the past years. Yet, numerous questions remain to be answered about which components should be added to IF and in which quantity depending on their metabolic fate and outcomes. Indeed, when it comes to human milk composition and infant nutrition in general, there is no “one-size-fits-all construct”.¹²⁷ Regarding metabolic health of infants, an improved IF would consist in modulating all macronutrients: proteins (to decrease the quantity but mostly improve their quality), lipids (to resemble the size, structure and composition of the fat globule by the addition of dairy lipids, cholesterol and MFGM, and also a balanced ω 3: ω 6 LC-PUFA ratio) and to supplement with prebiotics, probiotics or synbiotics. However, further studies are needed to improve IF composition and gain comprehension on how it may modulate the interplay between host metabolism and gut

microbiome and exert long-term health benefits. Gut microbiota development plays a key role but due to its complexity, the underlying pathways impacting the infant biology remain largely unknown. Animals such as the nonhuman primate and the neonatal piglet, excellent preclinical models for the human infant,^{93,249} proved to be useful to control and account for some confounding factors found in human studies and to investigate the mechanisms involved in the long-term effects of early nutrition. They allow for the screening of potential nutritional factors and the selection of the most promising ones. Yet there is still a need for a standardized model for infant growth and development. Besides, they remain models and additional well-designed longitudinal human studies are needed to investigate the effects of the IF composition on host metabolism beyond infancy.

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Conflicts of Interest

None.

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Figure legends**Fig. 1. Short- and plausible long-term effects of neonatal feeding**

BF, breastfed infants; CHD, coronary heart disease; FA, fatty acid; HMOs, human milk oligosaccharides; IF, infant formula; IGF-1, insulin-like growth factor-1; IR, insulin resistance; LDL, low density lipoprotein; MFGM, milk fat globule membrane; TD2, type-2 diabetes; TG, triglycerides

Fig. 2. Long-term metabolic health: the potential pathways involving gut microbiota

GLP-1, glucagon-like peptide-1; HMOs, human milk oligosaccharides; IGF-1, insulin-like growth factor-1; miRNAs, non-coding microRNAs; PYY, peptide YY; SCFAs, short-chain fatty acids

Figure 1

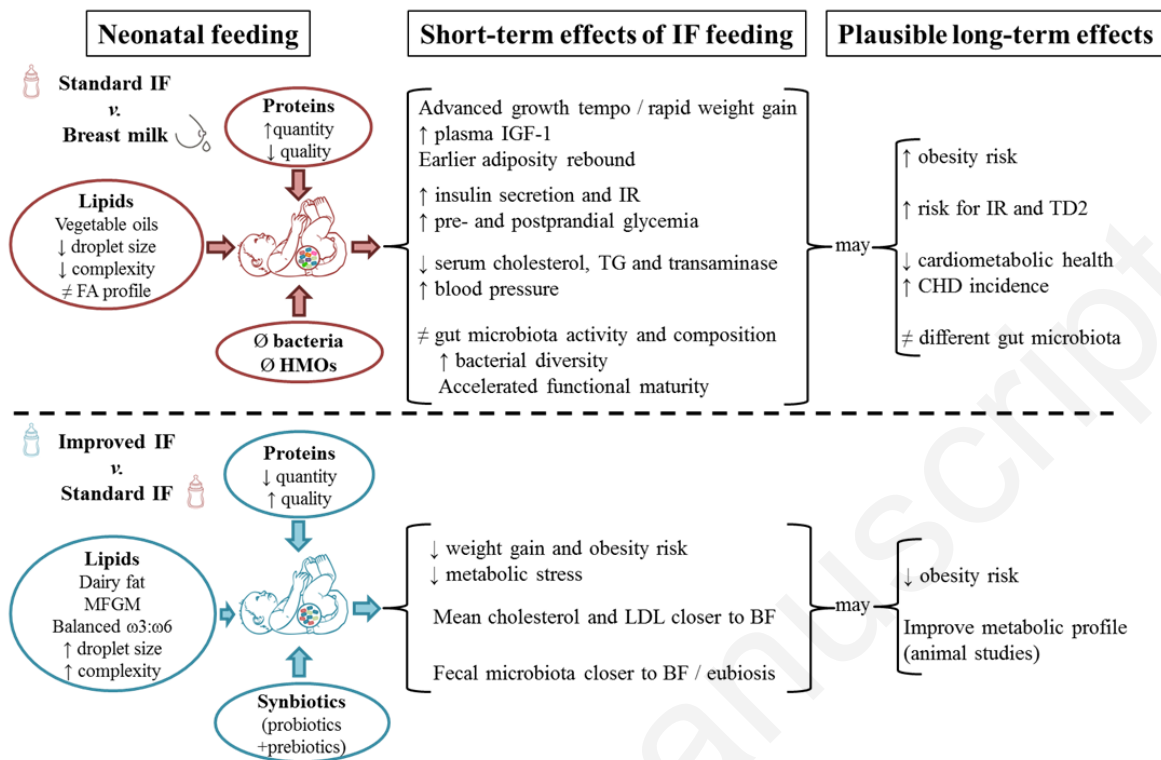


Fig. 1. Short- and plausible long-term effects of neonatal feeding

IF, infant formula; v., versus; FA, fatty acid; HMOs, human milk oligosaccharides; IGF-1, insulin-like growth factor-1; IR, insulin resistance; TG, triglycerides; TD2, type-2 diabetes; CHD, coronary heart disease; MFGM, milk fat globule membrane; LDL, low density lipoprotein; BF, breastfed infants

Figure 2

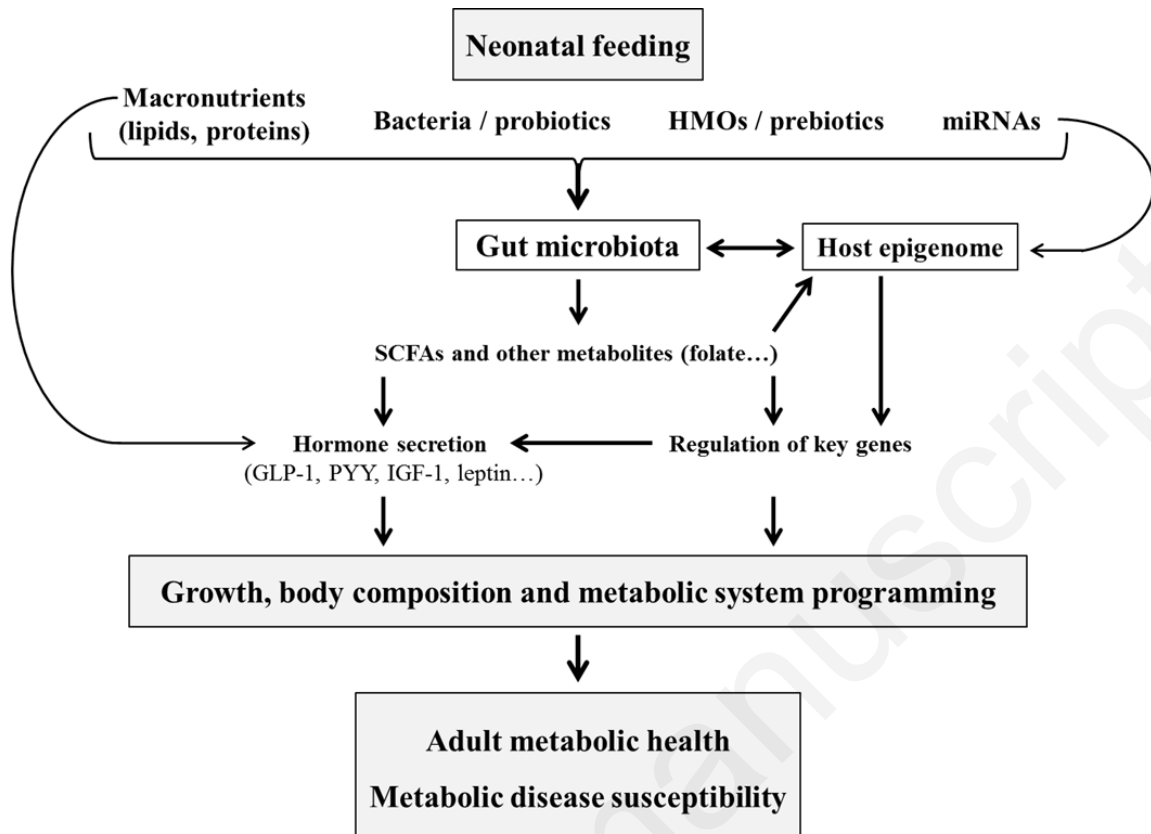


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