STUDY ON SAFETY AND EFFICACY OF INULIN AND OLIGOFRUCTOSE IN NEONATES.

Mariona Gispert Llauradó

Dipòsit Legal: T 50-2015

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European Thesis

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Study on safety and efficacy of inulin and oligofructose in neonates

EUROPEAN THESIS
Universitat Rovira i Virgili

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EUROPEAN THESIS

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CERTIFIQUEN:

Que aquest treball, titulat "Study on safety and efficacy of inulin and oligofructose in neonates", que presenta Mariona Gispert Llauradó per a l'obtenció del títol de Doctor, ha estat realitzat sota la nostra direcció al Departament de Medicina i Cirurgia d'aquesta universitat i que acompleix els requeriments per poder optar a Menció Europea.

Reus, 30 d'abril de 2014.

I per a que així consti, firmem aquest certificat.

Dra. Verònica Luque Moreno

Dra. Natàlia Ferré Pallàs

AGRAIMENTS

Amb aquestes línies m'agradaria donar les gràcies a totes aquelles persones que amb la seva ajuda han col·laborat en la realització d'aquest treball.

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LIST OF ABREVIATIONS

SCFA	Short-chain fatty acids
HMO	Human milk oligosaccharides
Glc	Glucose
Gal	Galactose
GlcNAc	N-acetylglucosamine
GOS	Galactooligosaccharides
FOS	Fructooligosaccharides or oligofructose
NSP	Non-starch polysaccharides
CO_2	Carbon dioxide
H_2	Hydrogen
FAO	Joint Food and Agriculture Organization
WHO	World Health Organization
SYN1	0.8g of Orafti® Synergy1/100 ml reconstituted milk
Control	Control formula infants
BF	Breasted infants
FF	Formula fed
SD	Standard desviation
IQR	Interquartile ranges
ISCED	International Standard Classification of Education

SUMMARY

Thesis title: Study on safety and efficacy of inulin and oligofructose in neonates.

Background: The newborn digestive tract is rapidly colonized after birth. Feeding type could influence this process. Infant formulas try to mimic the bifidogenic effect of human milk by addition of prebiotics. Although previous studies assessed the effects of prebiotics in newborns, none of these, to our knowledge, studied the effect of Orafti Synergy1 (oligofructose-enriched inulin) during the firsts 4 months of life.

Aim: To demonstrate the efficacy, safety and tolerance of a 0.8 g/dL SYN1 supplemented infant formula during the firsts 4 months of life.

Methods: In a double-blind, randomized, placebo-controlled and parallel trial, formula fed healthy term newborns were randomized to receive a control (controls) or SYN1 supplemented infant formula (SYN1). Breastfed newborns (BF) were also followed up for comparison. Anthropometry, water balance, blood parameters, formula intake and acceptance, digestive symptoms, adverse events, child's behaviour, infant's illnesses, atopic dermatitis, stools frequency and characteristics and faecal microbiota were assessed.

Results: A total of 252 formula fed infants were randomized at birth (124 controls, 128 SYN1) and 131 BF infants were recruited; after 4 months 68 controls, 63 SYN1 and 57 BF completed the study. SYN1 infants showed a

microbiota composition closer to that of BF infants, with a trend towards higher Bifidobacterium cell counts, softer stools and a higher deposition frequency compared to controls. There were no differences between formulas in any of the safety parameters such as growth, relevant adverse events, water balance or blood parameters.

Conclusion: A 0.8 g/dL SYN1-supplemented infant formula during the first 4 months of life is well tolerated, safe and effective, promoting a gut microbiota closer to that promoted by breastfeeding.

INTRODUCTION

INTRODUCTION

Breastfeeding is considered the gold standard, the optimum nutrition and protection for developing infants (1). There is compelling evidence that breast-feeding has positive effects on health in the newborn and in later life (2).

The World Health Organization recommends exclusively breast feeding for infants from birth to six months, and continue partially breastfeeding the infant up to the age of 2 years, during the introduction of complementary feeding (3;4). Although the majority of the mothers start breastfeeding their infant from the first day (50-75% of mothers), the percentage of exclusively breast-fed infants at 4 months is quite different (5).

In an attempt to maintain the breast milk benefits, the formula milk manufacturers try to offer products that could simulate the composition and biological effects of human milk as closely as possible (6;7).

A focus of research, driven by a commercial interest, is the development of some functional foods called prebiotics to confer benefits for the host health.
This dissertation focuses in the effect of a prebiotic-supplemented infant formula during the first 4 months of life in healthy term infant's population.

1.1. INTESTINAL MICROBIOTA

1.1.1. Definition/ Description of intestinal microbiota

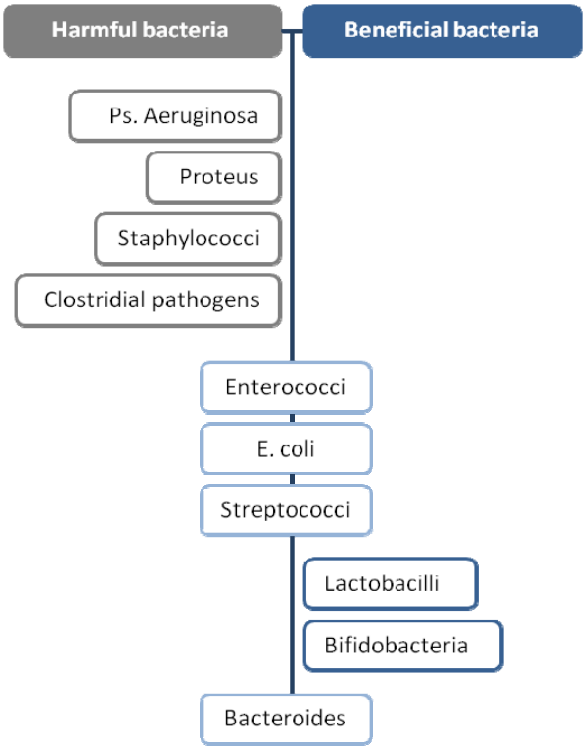
Microbiota refers to a *"population of microscopic organisms that inhabit a bodily organ or portion of a person's body"* (8). A recently published review claims that the microbial ecosystem of our body consists of approximately 100 trillion organisms (9;10) fit in 500-1000 different species (11).

The human intestinal microbiota can be described as a collection of a variety of microorganisms that reside in the gastrointestinal tract (9;11), The vast majority of these microorganisms inhabiting the large intestine are bacteria (9) although, archaea, viruses and protozoans are also present (11;12). There are ten times more bacterial cells in the intestinal microbiota than cells in the human body (9;13;14). The microbial community inhabits the human intestinal tract, being the large intestine the most colonized region of the digestive tract (15). It is characterized for having a high population density, wide diversity and complexity interactions with the human host (16).

Microbiota has metabolic, trophic and protective functions. The metabolic function includes the fermentation of non-digestible carbohydrates to produce short-chain fatty acids (SCFA), the synthesis of vitamins and the absorption of ions (17). Moreover, contributes in the maturation of the immune system (trophic function) and provides a barrier against colonization by pathogens (protective function) (18;19).

The mainly phyla found in the human gut are Firmicutes (e.g. *Lactobacillus, Clostridium, Staphylococcus, Enterococcus*), Bacteroidetes (e.g. *Bacteroides)*, Proteobacteria (e.g. *Escherichia, Enterobacteriaceae)*, Actinobacteria (e.g. *Bifidobacterium)*, Verrumicrobia, Fusobacteria and Cyanobacteria phyla (14;20). Most of the species are classificated into three categories depending on their effect on human health (Figure 1). *Lactobacillus and Bifidobacterium*, which are beneficial to health, other commensal bacteria like *Bacteroides*, which may have both positive and negative characteristics, and finally, some pathogenic bacteria such as *Clostridia*.

Figure 1. Harmful and healthy effects of predominant bacteria.

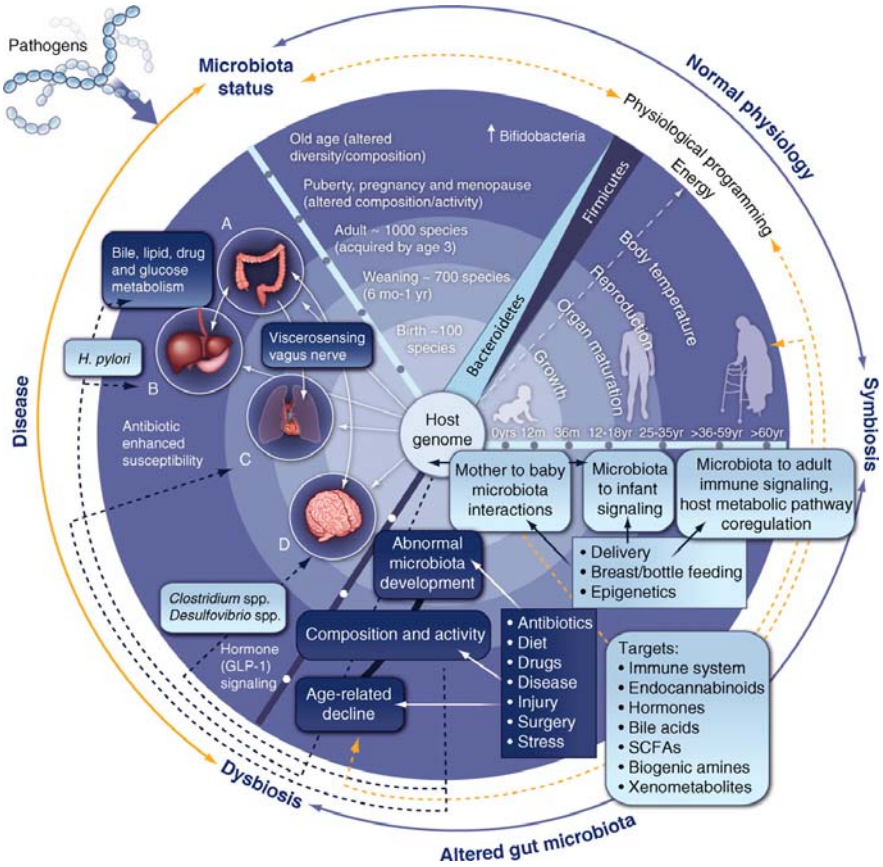


Adapted from Gibson 1995 (21) *Meyer 2009* (19).

It is well known that there are interindividual variations in the composition of the microbiota (22). This composition is also determined and can fluctuate by a wide variety of host's characteristics and processes (digestive secretions, digestive physiology, innate and adaptive immunity, genetic, disease, use of drugs and antibiotics and diet), microbiological relationships (metabolic cooperation, bacterial antagonism, competition for nutrients and adhesion sites) and environmental factors (geography, substrate availability, redox potential and local pH) (15).

In the recent decades it has been shown that the human intestinal microbiota can play a major role modulating the health and disease from birth to the old age. Some factors like antibiotic treatments, invasion by pathogens or some dietary changes can alter and disturb the gut microbiota causing a dysbiosis (also called dysbacteriosis, refers to microbial imbalance inside or on the body), which could be associated with Inflammatory Bowel Disease, Crohn's disease, ulcerative colitis and also increasingly linked to multiple sclerosis, diabetes and obesity (Figure 2) (10;23).

Figure 2. Influence of gut microbiota in human health.



This figure represents the effects of microbiota in human health, from birth to the old age. At birth the way of delivery, feeding type and some epigenetic factors are implicated in the microbiota development. Also some changes in the microbiota would occur if there were some disease, stress situation, changes in diet, antibiotics and drugs, among others. In the first months of life microbiota develops rapidly and changes occur from childhood to adulthood, being the Firmicutes the predominant specie in adulthood in contrast to the Bacteroidetes, which are predominant in childhood. Microbiota is also important because produces some energy for the body temperature regulation, reproduction, maturation and growth. And finally, some disruption of the microbiota (dysbiosis) may produce different diseases like inflammatory bowel disease, colon cancer and irritable bowel syndrome (A); gastric ulcers, some liver disease, obesity and metabolic syndrome (B); asthma, atopy and hypertension (C) and some mood and behaviour problems through hormone signalling (D). *Source: Nicholson, 2012* (23).

1.2. FUNCTIONAL FOOD

During the twentieth century the main goal of nutrition was to establish the nutritional recommendations needed to prevent deficits and excesses of some nutrients, looking for an "adequate nutrition" for each individual. It was at the end of the 20th and beginning of the 21st centuries, when the concept of "adequate nutrition" begun to be replaced by "optimal nutrition". Therefore, dietary intake should not only provide the basic nutritional recommendations, but also aims to maximize the physiological and psychological functions of each individual through nutrition, in order to ensure the welfare and health, while reducing the risk of disease (24-26).

In this scenario where optimal nutrition is desired, the concept of functional food appeared. Hippocrates, over 2000 years ago already stated "Let food be your medicine and medicine your food." At that time, the concept of functional food as we understand nowadays did not exist yet, but the terms food and health were already related.

The concept of functional food emerged in the 80s in Japan due to the increase of diseases related to lifestyle and the consequent increase in health care costs. In 1986, the Ministry of Health and Welfare created the Functional Food Forum, a commission of nutrition experts with the objective of improving the health of its citizens. As a result of this, in 1991, the Ministry of Health and Welfare was the first to allow the legal marketing of the functional foods with the name of "Foods for Specified Health Uses " (27-29).

There is no universal definition about the concept of functional food. The various international institutions have developed their own definitions (27). The European Commission's Concerted Action on Functional Food Science in Europe, coordinated by The International Life Sciences Institute proposed in 1998, the following definition of functional food:

"A food can be regarded as 'functional' if it is satisfactorily demonstrated to affect beneficially one or more target functions in the body, beyond adequate nutritional effects, in a way that is relevant to either an improved state of health and well-being and/or reduction of risk of disease. Functional foods must remain foods and they must demonstrate their effects in amounts that can normally be expected to be consumed in the diet: they are not pills or capsules, but part of a normal food pattern" (28).

From a practical standpoint, a functional food may be (27):

- A natural food.
- A food to which a component has been added.
- A food from which a component has been removed.
- A food in which the bioavailability of one or more components has been modified.
- Some combination of these possibilities.

A food can become a functional food by the use of any of the five methods listed below (24;27):

1. Removing a component that is known to cause a harmful effect when consumed (e.g. an allergenic protein).

2. Increasing the concentration of a natural compound naturally found in food, resulting in a higher than usual concentration, and if this induces a positive health outcome (e.g. the addition of calcium to milk).
3. Adding a component that is not commonly found in that food, but have been shown to provide beneficial effects (e.g. adding prebiotics in yogurt).
4. Replacing a component, whose intake is usually excessive (e.g. fat) to another component that has been observed beneficial effects on health (e.g. carbohydrates).
5. Increasing the bioavailability of a component which is known to produce a beneficial effect or hinder the bioavailability of harmful components (e.g. to increase the transferrin in milk and to decrease phytosterols).

The industry of functional foods is in development process. Currently, the majority of the population has the minimum nutritional recommendations covered, so that the consumers are increasingly interested in the consequences that food choices can have on their health. So that, food industry success strongly depends of consumers' acceptance of products, and particularly of the foods capacity to satisfy customers expectation. Menrad K already affirmed in 2003 that the functional food market in Europe would increase considerably, and could reach 5% of share of the food market in 10 years (30).

According to the conclusions of the Consensus Document from 1999 of The European Commission's Concerted Action on Functional Food Science

in Europe (28), the main objectives for the development of functional foods are: growth, development and differentiation, metabolism of substances, oxidative stress, cardiovascular system, gastrointestinal physiology and function and behaviour and psychological function.

Numerous substances have been described as functional foods, and some can currently be found on the market such as calcium enriched milk; foods enriched in vitamins A and D, another Ω-enriched dairy products to control the plasma cholesterol levels and cardiovascular disease; fruit juices with antioxidants, etc. And finally, another large group of functional foods are probiotics and prebiotics, being the last ones the starting point of our research.

1.3. PREBIOTICS

1.3.1. The concept of Prebiotic

The prebiotic term was first defined by Gibson and Roberfroid in 1995 as a *"nondigestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health"* (21). Later, due to the big academic and industrial interest of the last decade, in 2004 and in 2008 some adaptations were made in the definition of prebiotic (Table 1) (31).

Table 1. Development of the prebiotic concept.

Year 1995 (21)
'A non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health'
Year 2004 (32)
'A selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora that confers benefits upon host well being and health.'
Year 2008 (33)
'A dietary prebiotic is a selectively fermented ingredient that results in specific changes, in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health.'

Adapted from Roberfroid 2010 (31).

Due to the growing interest of the food industries to market this type of food, a prebiotic effect has been attributed to many components that actually do not fulfil all the necessary criteria (for example some carbohydrates). Prebiotics effectiveness comes from their ability to resist digestion and to reach the large intestine where, a group of microorganisms use them as the substrate for fermentation.

Criteria that a nutritional component must fulfil to be considered a prebiotic (34):

1. Resistance to gastric acidity, resistance to hydrolysis by mammalian enzymes and resistance to gastrointestinal absorption

Digestion is a complex process that involves chemical and mechanical degradation of complex molecules into smaller molecules that can be absorbed by intestinal epithelial cells. If we focus on carbohydrates, the transportation of them into the cells is limited to the monosaccharides, what means that all the complex carbohydrates and disaccharides need to be digested before being absorbed. The digestion is performed by enzymes such as amylases and disaccharidases that transform complex carbohydrates and disaccharides into monosaccharides such as glucose, fructose and galactose.

The absorption process is defined as an active or passive transfer of digestion products, vitamins, minerals and fluids through the gastrointestinal mucosa, travelling from the digestive tract to the blood or lymph. The absorption is carried out, mainly, in the small intestine

because the surface area of the intestine is increased due to the presence of villi and due to the position in brush border of the epithelial cells. In the large intestine the absorption of the additional water and some ions happens.

Monosaccharide absorption is performed by an active transport (together with sodium) in the case of glucose and galactose, and by a passive transport through facilitated diffusion in the case of fructose. Subsequently, after being absorbed through the intestinal epithelial cells, these molecules enter the portal circulation to reach the liver (35).

Not all carbohydrates can be digested and absorbed by the digestive system. It has been estimated that every day about 20-60g of intact carbohydrates reach the colon. These carbohydrates resistant to digestion in the small intestine are (15;36;37):

- Polyols: sorbitol and xylitol.
- Non-digestible oligosaccharides (NDO): raffinose, stachyose, fructo-and galactooligosaccharides, polydextrose and inulin.
- Non-starch polysaccharides (NSP) (dietary fibre): cellulose, hemicellulose, pectin, arabinoxylan, β-glucan, glucomannan, vegetable gums, mucilages and hydrocolloids.

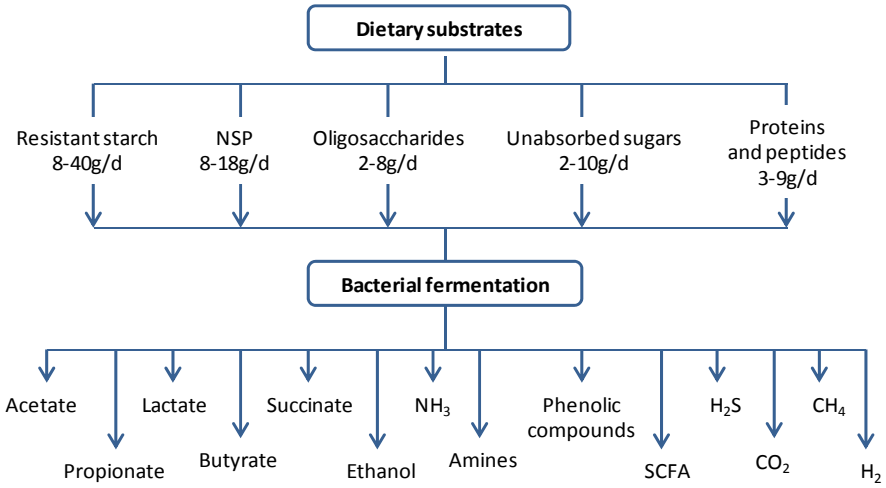
Given the objective of this thesis dissertation, we will focus in NDO. Inulin and oligofructose consist of fructose molecules linked by β-(2-1) fructosyl-fructose. Because of the beta configuration of the anomeric C_2 in their fructose monomers, it has the property of being resistant to digestion

enzymes and, therefore, resistant to the gastrointestinal absorption (38;39).

2. Fermentation by intestinal flora

Due to the property of prebiotics to escape to the enzymatic hydrolysis in the small intestine, they are selectively fermented by the microbiota in the colon (31). The fermentation of these carbohydrates by the colonic microflora is an anaerobic process that produces the short chain fatty acids (SCFA) acetate, propionate and butyrate; lactate, carbon dioxide (CO_2) and hydrogen (H_2), all of them feeding substrates for the gut flora (7;39-41). The main substrates of the bacterial flora are shown in Figure 3 (42).

Figure 3. Substrates and products involved in bacterial fermentation in the colon.



Adapted from Gibson 1996 (42).

It is estimated that the concentration of SCFA in the colon ranges between 70 and 100mmol/l (43). Acetate and propionate are absorbed and transported to the liver for the gluconeogenesis and lipogenesis, and butyrate is the principal energy source for the colonic epithelia. Eventually, the biomass is excreted through stools, and gases produced by bacteria's fermentation are excreted through breathing or through stools (44-46).

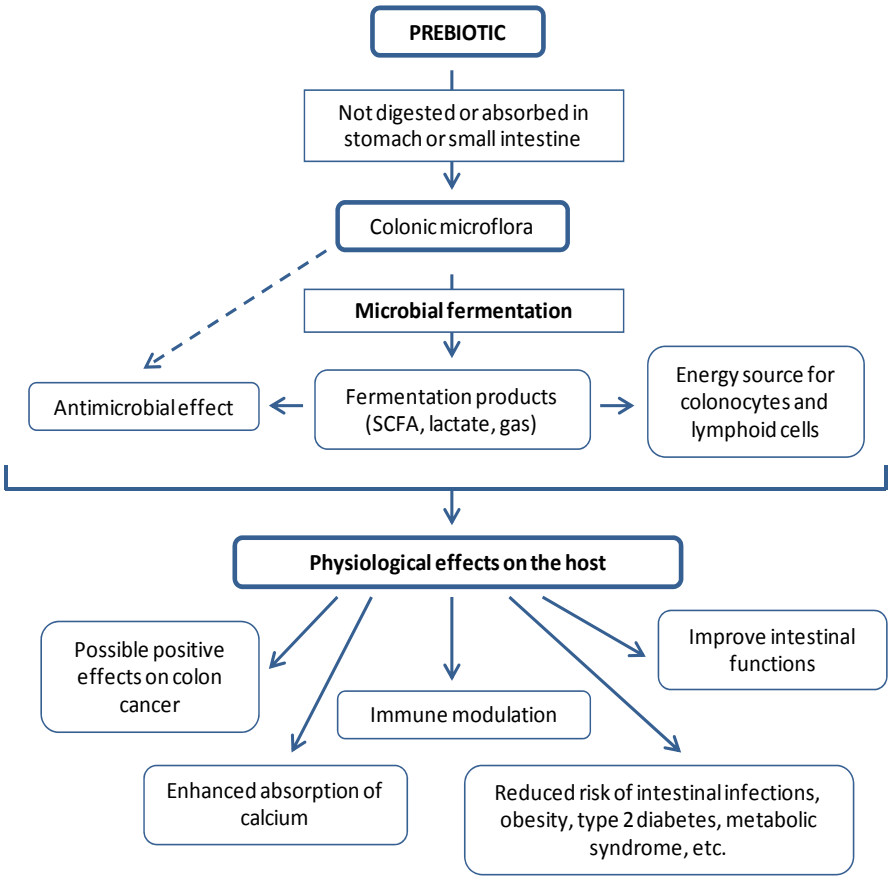
3. Selective stimulation of growth and/or activity of those intestinal bacteria that contribute to the health and welfare

SCFA are the main end products of the bacterial fermentation and contribute to lower luminal pH in the large intestine. Human studies showed that this acidification of the colon stimulates the development and growth of bifidobacteria and lactobacillus population, which are perfectly adapted with low pH, and also avoids the growth of pathogenic bacteria (7;43). This selective stimulation of bifidobacteria and lactobacillus produce some positive effects on human health, which are detailed in the next section.

1.3.2. Effects of prebiotics on human health

It has been known that prebiotics have positive effects on human health. The main effects that have been attributed to prebiotics are explained below and summarized in Figure 4.

Figure 4. Main effects of prebiotics in the intestinal tract.



Adapted figure from Sherman (47) *and Meyer* (19).

The most important effects of prebiotics in human health are:

- ✓ Improvement and/or stabilization of gut microbiota composition and stimulation of beneficial microbial activities, through selective fermentation

And

- ✓ Improvement of intestinal barrier functions and increased resistance to colonization by enteric pathogens.

As I mentioned before, due to the property of prebiotics to escape to the enzymatic hydrolysis in the small intestine, they are selectively fermented by the microbiota in the colon (31). The main end products of the bacterial fermentation are the SCFA, which contribute to lower luminal pH in the large intestine. This acidification of the colon stimulates the development of bifidobacteria and lactobacillus, which are perfectly adapted to such low pH, and also avoids the growth of pathogenic bacteria (43).

- ✓ Improvement of intestinal functions (stools bulking, regularity and consistency).

Prebiotics increase the gas production and the microbial mass in the colon, and this produce an increase in the faecal content. Furthermore, SCFA produced through the fermentation are absorbed by the colonocytes, who stimulate the growth and the absorption of salts and water. This fact increases the humidity of the faecal bolus and the intestinal motility. So, the transit time and the time available to water reabsorption decreases. Summarizing, prebiotics produce an increase on faecal bolus weight and soften its consistency (41). However, this

fermentation can have a clinical disincentive: the CO_2 and H_2 produced through the fermentation can induce unwanted symptoms as increased gas production (48).

- ✓ Increase of mineral absorption (calcium and magnesium) and improvement of bone health.

Because of the few studies in humans and the lack of knowledge with any certainty, there are different hypotheses about the mechanism through which prebiotics could enhance mineral absorption (49). Firstly, as a result of the prebiotic fermentation, a high concentration of SCFAs reduces the pH of the colon. This fact may increase the solubility and availability of some minerals, specially magnesium and calcium. Another hypothesis is that prebiotics may increase the water content in the colon, increasing the solubility of some minerals. And finally, the SCFAs may stimulate the proliferation of epithelial cells, enhancing the absorption capacity through the colonocytes (31;49-52).

- ✓ Modulation of gastrointestinal peptides production, energy metabolism and satiety, obesity, type 2 diabetes, metabolic syndrome, etc.

Recent investigations suggest that the composition of intestinal microflora may play a role in the obesity development and its related disorders, due to the discovery of differences in gut microbial composition between obese and lean individuals, and also between diabetic and non-diabetic individuals (53-55).

Regarding the possible satiating effect of prebiotic supplementation, there have been controversial results. To our knowledge, in three of the five published trials (56-60), prebiotics produced a significant satiogenic effect in human subjects. The proposed mechanism to promote satiety might be an increase in peptide YY (57;59;61), glucagon-like peptide 1 (57;62), glucagon-like peptide 2 concentrations (63), and reduction in ghrelin concentrations (61) induced by prebiotic supplementation.

There is no consensus about the effect of prebiotic supplementation on body weight. Significant reductions in body weight were found in two studies (61;64), but no changes in body weight were reported in other three trials (65-67). Moreover, a non-significant effect of prebiotic was observed in a recent meta-analysis (68).
On other hand, three studies found a significant reduction in total energy consumption during the prebiotic supplementation compared to placebo, but these results were not significant in a meta-analysis (56;61;65;68). Therefore, more studies are necessary to demonstrate an effect of prebiotics supplementation reducing energy intake.

Other effect that some researchers found was an improvement of postprandial glycaemia in normal weight and obesity participants after a prebiotic supplementation (57;69), and also a meta-analyses support this property (68). Furthermore, a statistically significant reduction in postprandial insulin concentrations were observed in overweight and hypercholesterolaemic participants (61;68;70). But, not significant effect of prebiotics on triglyceride concentrations in healthy, overweight or hypercholesterolaemic subjects (68).

- ✓ Immune system modulation, regulatory functions and reduction of risk of intestinal and respiratory infections.

Gastrointestinal microbiota might modulate the physiology of the barrier function and also the immunology system and inflammatory responses (71). Prebiotics produce a selective increase/decrease in the number of bacterial species and change the collective immune interactive profile of the microflora. Also the products of the fermentation, the SCFA, may interact changing the activity of the immune cells and enterocytes. Finally, another hypothesis is that the butyrate may reduce the epithelial cells requirement of glutamine, leaving this glutamine for the immune system cells (31). Due to the capacity of prebiotics to inhibit the adhesion of pathogenic bacteria in the epithelial surfaces, this produces a protective effect against gastrointestinal infections (72;73).

- ✓ Reduction of colon cancer risk.

Through the fermentation process, prebiotics increase the beneficial bacteria, the SCFA and also prevent the pathogenic bacteria growth in the colon. Thus, it reduces the production of carcinogenic substances. Moreover, the biomass and the faecal bolus are increased, this produce more acceleration in the colonic transit time and the microflora is exposed less time to potential carcinogenic agents (41).

1.3.3. Candidate ingredients to be considered prebiotics

Among the numerous food ingredients, some carbohydrates, peptides, proteins and lipids are candidates to be prebiotics (21). However, most of the interest in the development of prebiotics is focused on the non-digestible oligosaccharides. These NDO are considered to be the most important prebiotic substrates (74).

Table 2. Summary of accomplishment prebiotic criteria of several oligosaccharides.

Carbohydrate	Nondigestibility (criteria 1)	Fermentation (criteria 2)	Selectivity (criteria 3)	Prebiotic status
Inulin	Yes	Yes	Yes	Yes
Oligofructose	Yes	Yes	Yes	Yes
Galacto-oligosaccharides	Probable	¿?	Yes	Yes
Lactulose	Probable	¿?	Yes	Yes
Isomalto-oligosaccharides	Partly	Yes	Promising	No
Lactosucrose	NA	NA	Promising	No
Xylooligosaccharides	NA	NA	Promising	No
Soybean oligosaccharides	NA	NA	NA	No
Gluco-oligosaccharides	NA	NA	NA	No

NA: data not available; ¿?: preliminary data, but still need further research. *Adapted from Roberfroid 2007* (34).

(Table 2) (34;35). Although the first criteria for classification as a prebiotic (nondigestibility) is not fully met, galactooligosaccharides (GOS) and lactulose still can be classified as prebiotics considering the existing data in human studies (32;34;75). Data are promising, but more studies are needed to confirm their effect. Especially, data are needed to confirm compliance with the first criteria of resistance to gastric acidity, resistance to hydrolysis by mammalian enzymes and resistance to gastrointestinal absorption, because is the criteria on which there is more lack of studies.

1.4. INULIN AND OLIGOFRUCTOSE

1.4.1. Introduction to inulin and oligofructose concept

Carbohydrates can be classified according to their molecular size, determined by the degree of polymerization, the type of bond and the characteristics of monomers, as proposed by the Joint Food and Agriculture Organization (FAO) and the World Health Organization (WHO) in 1997 (Table 3) (76).

Table 3. Classification of carbohydrates.

Class (DP)	Subgroup	Principal components
Sugars (1-2)	Monosaccharides	Glucose, fructose, galactose
	Disaccharides	Sucrose, lactose, maltose, trehalose
	Polyols (sugar alcohols)	Xylitol, erythritol, isomalt, maltitol
Oligosaccharides (3-9)	Malto-oligosaccharides	Maltodextrins
	Non-α-glucan oligosaccharides	Raffinose, stachyose, fructo and galacto oligosaccharides, inulin*
Polysaccharides (≥10)	Starch (α-glucans)	Amylase, amylopectin, etc.
	Non-starch polysaccharides (NSPs)	Cellulose, hemicellulose, pectin, arabinoxylans, β-glucan, glucomannans, etc.

DP: degree of polymerization. *Our product study. *Adapted from Cummings, 2007* (36).

Although this classification divides the carbohydrates in three groups depending on the degree of polymerization, some carbohydrates like inulin exists in nature in various molecular forms, so the IUB-IUPAC Joint Commission on Biochemical Nomenclature stated that the borderline

between oligosaccharides and polysaccharides can't be drawn too strictly (35;77).

Focusing on the oligosaccharides group, we find the maltodextrins, which are widely used in food industry to substitute fat, to modify the texture of the products and as sweeteners. The other group are the non-α-glucan oligosaccharides, among which we highlight inulin and fructooligosaccharides (36). Maltodextrins are digestible carbohydrates, whereas inulin and fructooligosaccharides pass through the upper gastrointestinal system without being hydrolyzed and then, reach the colon where they stimulate the development of the bifidus-predominant flora (78). So they are called "non-digestible oligosaccharides" (NDO) (36;79) and also they have the basic characteristic of being soluble and fermentable dietary fibre, and should be classified and labelled as such (39;80).

1.4.2. Natural occurrence of inulin

A German scientist was the first, in 1804, in discover and isolate this carbohydrate from *Inula helenium*, which was called inulin (81). Inulin and oligofructose are natural food ingredients widely found in many plants, vegetables, fruits and cereals including leek, onion, wheat, garlic, banana and chicory (82;83) (Table 4). It has been estimated that the average daily consumption of inulin and oligofructose in Europe is between 3-10g/day and between 1-4g/day in America (84).

The most important source of inulin and oligofructose for the food industry production is essentially the chicory. Chicory is a biennal plant

usually with bright blue flowers. It is cultivated for salad leaves, blanched buds or for roots (Figure 5). It lives in Europe, North America and Australia.

Table 4. Inulin and oligofructose content in most common source plants.

Source	Edible parts	oligofructose	Inulin
Onion	Bulb	3.1	1-7.5
Jerusalem artichoke	Tuber	58.4	17-20.5
Chicory	Root	3.9	15-20
Leek	Bulb	0.9	3-10
Garlic	Bulb	3.9	16
Artichoke	Leaves-heart	2.4	2-7
Banana	Fruit	1.4	±1
Barley	Cereal	-	0.5-1
Dandelion	Leaves	-	12-15
Salsify	Root	-	±20
Wheat	Cereal	-	1-4

Source from Van Loo J 1995 (84) *and Dumitriu S 2005* (85).

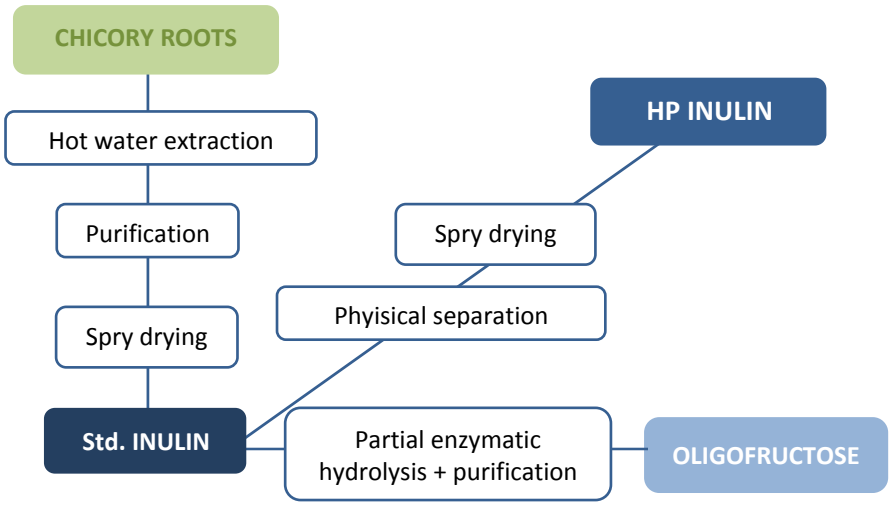
Figure 5. Chicory plant and root.

On left: blue flowers; on right: blanched buds and roots.

1.4.3. Industrial production of inulin and oligofructose

During the last two decades food industry tried to isolate and purify the inulin and oligofructose naturally present in some vegetables and plants, to use it as a functional ingredient (86). Nowadays it is possible to use inulin and oligofructose in their pure form. The industrial production of inulin and oligofructose involves different phases that are summarized in Figure 6.

Figure 6. Industrial production of inulin and oligofructose.



Std.: Standard. HP: High performance. *Adapted from Roberfroid 2005* (35).

The production of inulin and oligofructose starts with the extraction of inulin from chicory roots by a hot water removal method, followed by a purification, evaporation and spray-drying phase. So it is obtained **standard inulin**.

Food industries also produce a high-molecular-weight inulin-type fructan or a long-chain inulin known as **HP inulin (High performance inulin)**, by applying specific separation techniques and removing the shorter-chain molecules.

And finally **oligofructose** is obtained by a partial enzymatic hydrolysis of the inulin. Oligofructose can be produced by two techniques, the last mentioned, and also can be synthesised from sucrose by transfructosylation (82;86).

1.4.4. Chemical structure and physical properties of inulin and oligofructose

From a chemical point of view, **standard inulin** is a polydisperse carbohydrate consisting mainly, but not exclusively, of β(2-1) fructosyl-fructose linkages between fructose molecules. The linear chain of inulin is either an α-D-glucopyranosyl-[β-D-fructofuranosyl]$_{n-1}$-β-D-fructofuranoside ($G_{py}F_n$) or a β-D-fructopyranosyl-[β-D-fructofuranosyl]$_{n-1}$-β-Dfructofuranoside ($F_{py}F_n$). These two compounds are included under that same nomenclature (Figure 7). The chain length range from 2 to 60 units, with an average degree of polymerization around 10 (34;38;82).

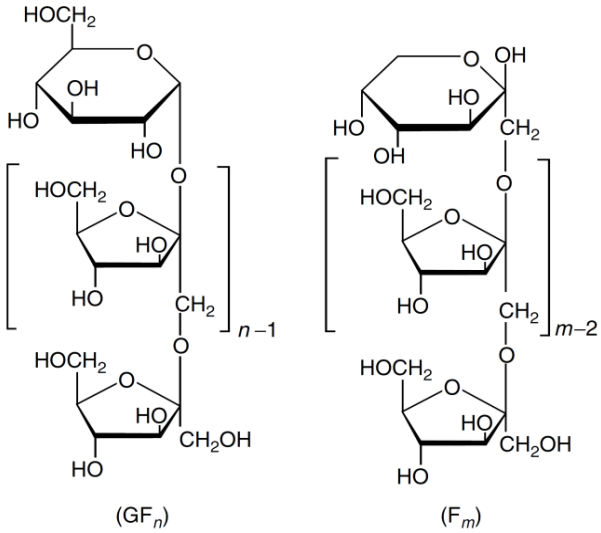
HP inulin is a long-chain inulin. It is a mixture of molecules with a degree of polymerization (DP) ranging from 10-11 to 60 and an average DP of 25 (34;38;82).

Oligofructose is a mixture of both $G_{py}F_n$ and $F_{py}F_n$ molecules. This can be obtained by the partial enzymatic hydrolysis of inulin using an endoinulinase (EC 3.2 1.7), in which the DP varies from 2 to 7 (average = 4)

(38;86). Or can otherwise be obtained by enzymatic synthesis using the fungal enzyme β-fructosidase from *Aspergillus niger*, in which the DP varies from 2 to 4 with a DP average of 3.6, and all oligomers are of $G_{py}F_n$ (34;38).

Both inulin and oligofructose contain β(2-1) linkages between the fructose molecules, and these linkages prevent from being digested as typical carbohydrates (82).

Figure 7. Chemical structure of inulin.



Source from Stephen AM 2006 (83).

Regarding the physical properties, inulin has a neutral taste without any flavour and oligofructose has a moderately sweet taste. Although inulin is sweeter than HP inulin, and it is often used to combine with other ingredients without modifing the flavours, the sweetest product is the oligofructose with 35% of sweetness compared to sucrose. Inulin is less soluble than oligofructose (10% instead 80% in water at room

temperature). Concerning the functionality in foods, inulin has the capacity to replace fat whereas oligofructose is used as a sugar replacer (86). All their physico-chemical properties are summarised in Table 5.

Table 5. Physical and chemical characteristics of inulin and oligofructose.

	Standard Inulin	HP inulin	Oligofructose
Chemical structure	GFn (2≤ n ≤ 60)	GFn (10≤n ≤ 60)	GFn + Fn (2≤ n ≤ 7)
Average DP	12	25	4
Dry matter (%)	95	95	95
Inulin or oligofructose content (%)	92	99.5	95
Sugars content (%)	8	0.5	5
pH (105 w/w)	5-7	5-7	5-7
Appearance	White powder	White powder	White powder
Taste	Neutral	Neutral	Moderately sweet
Sweetness (v. sucrose)	10%	None	35%
Solubility in water (g/l)*	120	25	>750
Viscosity in water	1.6	2.4	<1
Functionality in foods	Fat replacer	Fat replacer	Sugar replacer
Synergism with	gelling agents	gelling agents	intense

G=glucosyl unit; F=fructosyl unit. DP: degree of polymerization. *at 25ºC, ** at 10ºC. *Adapted from Franck A 2002* (86).

1.4.5. Food applications of inulin and oligofructose

As mentioned before in the functional food chapter, the consumers are increasingly interested in the consequences that food choices can have on their health and they demand foods with great taste and if it's possible, foods that provide health benefits (82).

Inulin and oligofructose have many nutritional, technological and health-promoting properties and are widely used in functional foods.

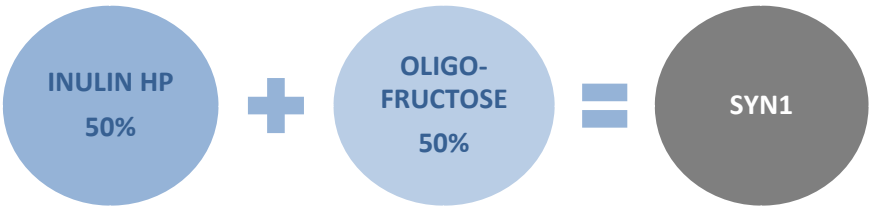
Oligofructose is used mixed with intensity sweeteners to replace sugars providing a great taste and masking the aftertaste of aspartame or acesulfame k. It is usually use in dairy products, baked goods, frozen desserts, low fat cookies and granola bars (86).

Because of the gelling characteristics, inulin is also used in dairy products, table spreads, baked goods, cream cheeses, processed cheeses, frozen desserts and dressings replacing the fat content. For example, in dairy products, inulin improves the flavour and the creamier mouthfeel (82). Due to the non-digestibility by the intestinal microflora, inulin and oligofructose are used as ingredients in the diabetic's food products. Furthermore, both inulin and oligofructose are used as a fibre ingredient in food products. It provides better viscosity and taste and it is an imperceptible way to add fibre in foods. As we mentioned before, both products have effect on intestinal function, increasing stools frequency, stools weight and reducing the pH level (82).

1.4.6. Orafti Synergy 1®

There are some commercial products that are mixtures of inulin and oligofructose, for example, the **Synergy 1® (SYN1)**. Orafti Synergy 1® is *"an enriched chicory inulin powder containing a carefully selected degree of polymerization distribution. Is a combination of chicory inulin molecules with selected chain lengths, enriched by a specific fraction of oligofructose produced by partial enzymatic hydrolysis of chicory inulin"*.

Figure 8. Composition schema of Orafty synergy 1.



1.5. EFFECT OF PREBIOTICS/NON-DIGESTIBLE CARBOHIDRATES IN NEWBORNS

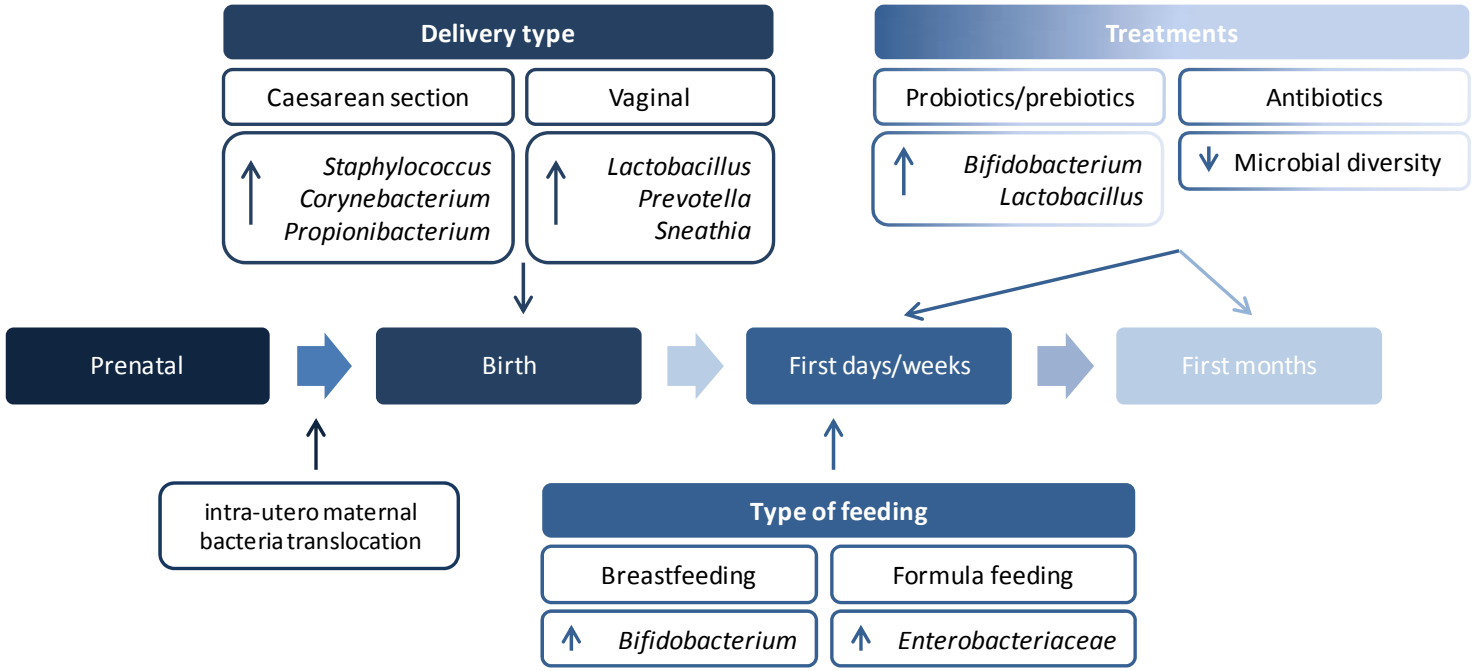
1.5.1. Development of intestinal microbiota in infants

Prenatally, the gastrointestinal tract is not completely sterile. There is some evidence suggesting a prenatal colonization of meconium, possibly through translocation of the mother's gut bacteria via the bloodstream (87). After birth, massive and rapid colonization of the digestive tract by microorganisms occurs as a part of the adaptation to extra uterine life (11;88).

First, the gut is early colonised by aerobic and facultative anaerobes such as Enterobacteria, Coliforms, Lactobacilli and Streptococci; followed on the 2^{nd}-3^{rd} day , by strictly anaerobic bacteria, such as Bifidobacteria, Bacteroides, Clostridia and Eubacteria, when the gut becomes an anaerobic environment (87;89).

This colonization process is modulated by factors such as type of delivery, mother's microbiota, hygienic measures, prematurity, antibiotic therapy and feeding type (Figure 9) (88;90).

Figure 9. Factors influencing the colonization process of the newborn.



Adaptded from Matamoros, 2013 (87).

Regarding the way of delivery, infants born by caesarean section have a different microbiota composition as compared with vaginally delivered infants. The last infants acquire their initial intestinal microbiota from vaginal and faecal microbiota of the mother (88). Differently, infants born by caesarean section show a different pattern of gastrointestinal microbiota, and are exposed initially to bacteria from the healthcare workers and hospital environment (90;91). Infants born by caesarean section are more often colonised with higher proportions of clostridia and bacteroides and less proportion of bifidobacteria and lactobacilli (6;7).

Concerning the feeding type, the intestinal microbiota of breastfed and formula-fed infants are comparable for the first three or four days but some differences appear over this time (6;8). Numerous studies have shown that the intestinal flora of infants fed with human milk includes higher proportions of Bifidobacterium and Lactobacillus than those of formula fed infants, who have more complex flora with higher proportions of Bacteroides, Enterobacteriaceae and Clostridium (47;92).

It has been known that *Bifidobacterium* is the dominant bacteria in the infant gut microbiota, but the presence of this microorganism is unstable and may change rapidly (87). The sucession of bacterial species is a very complex process, so microbiota varies during the first months of life. When the infant reaches 1-2 years of age, it establishes and at 3 years old begins to resemble the adult microbiota (11;87).

1.5.2. Human Milk Oligosaccharides

Human milk oligosaccharides (HMOs) represents the third largest component in the human milk, after lactose and lipids (93). Almost 200 oligosaccharides have been identified in the human milk (94), but they are characterized by an enormous structural diversity (95). Structurally, HMOs are composed by combinations of five monosaccharide: glucose, galactose, n-acetylglucosamine, fucose and sialic acid (96).

Each women synthesize different kind of oligosaccharides (97), and moreover, there are some variations in the composition and concentration over the course of lactation process (98-101). Human milk contains oligosaccharides at a concentration of 20–25 g/L in the colostrums and when production matures, its concentrations decline to 5–20 g/L (102;103).

These oligosaccharides are not digestible by intestinal enzymes. Therefore, intact HMOs reach the large intestine and they are fermented to SCFA and lactic acids. This makes them to be recognized for its “bifidogenic” (ability to increase Bifidobacteria content) or prebiotic effect on the gut microbiota (96;98;104). HMOs have several key beneficial effects on the development of the neonatal intestine, stimulating the growth of probiotic bacteria, having anti-adhesive properties to protect the epithelial surface in front of pathogenic infections and also having an effect on the development of the immune system (6;98;100;105).

Already, in 1905, Moro reported the predominance of Bifidobacteria in the breast-fed infants microbiota compared to formula-fed infants (106). It was in 1974 when it was suggested the effect of some molecules from human milk defined as "bifidus factor" to promote this bacteria growth (107). Nowadays it has been known that most of the differences in the bacterial colonization between breast-fed and formula-fed infants can be explained by these molecules, the HMOs (108).

An in vitro assay with human breast milk samples was performed to determine the kinetics of bacterial growth and the HMOs consumption by 12 bifidobacterial strains. The highest growth was observed with *Bifidobacterium longum infantis* strains, which consume nearly all HMOs, compared with other strains tested like *Bifidobacterium longum adolescentis, breve and bifidum*, which showed lower or moderate growth hability (109). Another study was performed with 57 healthy mothers and their infants. Breast milk samples were collected and processed in vitro with HMOs. As in the previous study, *Bifidobacterium longum infantis* was the fastest strain and it consumed all HMOs incorporated in their intact forms. These results propose a symbiotic effect between HMOs and microbiota (110).

Because of the benefits on infants' intestinal microbiota attributed to, researchers are interested in the promotion of similar effects to formula-fed infants. However, in contrast to human milk, cow's milk only contains trace amounts of oligosaccharides. Due to the variety, variability and complexity of the HMOs, identical structures like HMOs are not available to reproduce in infant formulas (111;112). To achieve this objective, non-milk oligosaccharides should be considered (100). There are some non-

milk oligosaccharides, like galacto-oligosaccharides (GOS) and fructo-oligosaccharides (FOS) that can be used as functional ingredients in infant formulas to partially mimic the functional properties of human milk oligosaccharides (112).

1.5.3. Effects of inulin and/or oligofructose in newborns

Regarding the nomenclature, there are some controversial opinions about the inulin and oligofructose terms depending on the authors, and in some studies the term oligofructose can be used to identify both compounds (32;38). Moreover, fructan is a general term used for *"any compound where one or more fructosyl-fructose linkages constitutes a majority of linkages"* (113). So, inulin and oligofructose have also been defined as fructans (35).

The beneficial effects of inulin and/or oligofructose on the intestinal microbiota have already been demonstrated in adults. A recent review reported significant bifidogenic changes in the composition of the adult microbiota after inulin or oligofructose consumption (19). Most of the studies showed an increase in Bifidobacterium bacteria after a prebiotic supplementation, however no consensus about the effect of prebiotics on other types of bacteria, was found (114-117). In infants, many studies examined the use of formulas supplemented with long-chain fructan polysaccharides (i.e., long-chain inulin and fructooligosaccharides (FOS)) and galactooligosaccharide mixtures (GOS) in healthy newborns (118-120).

To study the safety and/ or efficacy of an infant/ follow-on formula, it is important to analyse the effect of such formula on safety and efficacy parameters (121). In the case of long-chain fructan polysaccharides supplemented formula, main outcomes to be analyzed would be:

For tolerance:

- Excessive gas production: the fermentation in the gut of a prebiotic enhances gas production and this could lead to a higher digestive discomfort and belly pain.

For safety (potential harm effects):

- Growth: it should be compared to a non-supplemented formula and breastfeeding (the gold standard).
- Hydration parameters: an increase in stools frequency and a watery composition of these stools could lead an immature infant to dehydration.

Efficacy (potential/ desired benefits on health and/ or well-being):

- Stools consistency and frequency: it has been reported that breastfed infants suffer less constipation than formula fed infants (122) mainly due to the HMOs.
- Gut microbiota.

All the parameters should be compared between the supplemented formula, a non-supplemented formula (placebo) and breastfeeding (as a gold standard reference with the desired effects).

To describe the state of the art in relation to the effects of prebiotics in newborns, we will consider all of the above mentioned outcome parameters in order to identify research needs.

Regarding growth parameters, some studies reported the weight gain (g/day), length and head circumference gain (cm/week) of the infants in prebiotic and control formula group (119;120;123-129). In a prospective study 110 healthy term infants were randomly assigned to 5 different feeding groups (control formula, standard formula enriched with SYN1 0.4g/dL, standard formula enriched with SYN1 0.8g/dL, standard formula enriched with GOS:FOS 0.8g/dL or breastfed group). The results showed no differences in weight, length and head circumference gain during the period of the study (1 month) (129).

No individual study reported significant differences in weight, however a meta-analysis of most of the previous mentioned studies showed that the weight gain increased significantly in the prebiotic-supplemented group (1g/day) compared to control formula (130). Also, two other meta-analyses have shown that prebiotic supplementation is associated with slightly greater weight gains (0.93g/day and 1.07g/day, respectively) (71;131). These results show that prebiotics have no negative effects on weight gain and allow a proper growth of newborns. Furthermore, no significant effects of prebiotics were observed in length and head circumference gain of infants in a recent meta-analysis (130).

A systematic review and commentary by the ESPGHAN concluded that the supplementation of infant formula with prebiotics has no adverse effects on growth in healthy, term infants (132) and also a Cochrane Review

concluded that the prebiotic supplementation of infant formula had no consistent effects on infant growth (71).

Concerning the stools frequency and consistency, a meta-analyses of four studies (119;123;125;133) concluded that prebiotics in infant formulas increase significantly the stools frequency compared to control formula (130) and also some studies found that the stools from the prebiotic group were significantly softer compared to control group (123;125-127;133). Also a prospective study, not included in the previous meta-analyses, was performed with 160 healthy term infants. Infants were randomly assigned to receive standard formula with 0.4g/dL GOS/FOS (90% galactooligosacharides and 10% long-chain inulin) or control formula (standard formula) during the first 12 weeks after birth. The results showed significantly higher frequency stools and also softer stools in prebiotic formula group compared to control group (120).

In relation to digestive tolerance, a systematic review performed by Rao et al. showed no difference between prebiotic and control formula group in the incidence of colic, regurgitation, vomiting and reported crying (131). Only a study showed that infants fed by a prebiotic supplemented formula (based on GOS and lactulose) had a higher risk of irritability, eczema and diarrhoea (125).

It has been shown in some studies that prebiotics could affect the infant's immunity, however there were no consistent data and further studies would be necessary.

To investigate the effect of a prebiotic mixture in the incidence of atopic dermatitis, it was performed a prospective trial with 259 health term infants with risk for atopy. Infants received a hypoallergenic formula with extensively hydrolysed cows' milk whey protein supplemented with 0.8g/dL of GOS/FOS or a standard hypoallergenic formula with extensively hydrolysed cows' milk whey protein without any supplementation, during the six month of study period. During this period, 10 infants developed AD in the prebiotic group and 24 in the control group. The authors suggested a beneficial effect of prebiotics in the development of atopic dermatitis in high risk population (93).

In a multicenter and prospective study with 342 healthy term infants assigned to the GOS/FOS formula or control formula (134) it was observed a lower incidence of gastrointestinal infections during the first year of life. In this trial, the group of infants supplemented with the prebiotic, also had lower respiratory infections, but this difference was not significant (134).

In the case of the Bifidobacteria counts, the results of four studies showed that the infant formula with prebiotics significantly increased these microorganisms in fecal samples (log10CFU/g stools) (126;127;135;136). In most of the studies, the milk is supplemented with galactooligosaccharides (GOS), instead oligofructose (FOS) or inulin. Only one of the four studies included in a recent meta-analyses publication (130) was performed with HP inulin. It was a clinical trial with thirty two term infants that were recruited and randomized to a supplemented formula with 0.8g/dL GOS/HP inulin mixture or standard formula without supplementation. Stool samples were collected at initiation of study formula feeding and 28 days later. The number of bifidobacteria at the

beginning of the study did not differ between feeding groups. However, after 28 days, bifidobacteria counts were significantly higher in the supplemented formula group compared to control group. A limitation of this study was the short-term follow-up of the children, which only lasted 28 days (135). However, another study with prebiotic supplementation (FOS) showed no significant differences in bifidobacteria counts depending on the formula group (137).

Another healthy bacteria to be considered is Lactobacillus. A recent meta-analyses showed that prebiotics significantly increased lactobacillus counts in faecal samples compared to the control formula group (126;130;136). As we previously mentioned, Bacteroides and Enterobacterias are considered neither harmful or pathogenic nor beneficial for health promotion, because they have positive and negative functions in human health. Related to this, Brunser et al. showed that there were no significant differences in the number of Enterobacteria and Bacteroides between the prebiotic and control formula groups (137).

These are the main effects of infant formulas supplemented with prebiotics like inulin, HP inulin, oligofructose or galactooligosaccharides (GOS) in healthy newborns. If we focus on the effects of our prebiotic product, SYN1, we only found a recent study in a term newborn infants (129). It is randomized, controlled 4-week trial in newborns, and infants were randomly assigned to 5 groups: control formula, standard formula enriched with SYN1 0.4g/dL, standard formula enriched with SYN1 0.8g/dL or standard formula enriched with GOS:FOS 0.8g/dL or breastfed group. Both SYN1 and GOS:FOS formulas promoted stools consistency and microflora composition closer to those of breastfed infants as compared

to a control formula. In a consistency score ranging from 1 (watery stools) to 4 (hard stools), the breastfed group had a consistency slightly above 1, the two supplemented groups about 1,5 and the control group about 2,5. The mean number of bifidobacteria was around 12% higher among the breastfed infants and the supplemented formulas as compared to the control group (129). To find out the effective dose of SYN1-supplemented formulas, the same study compared the 0.8 g/dL with a 0.4 g/dL SYN1-supplemented formula; they found that the 0.8g/dL supplemented formula leaded to a higher number of depositions and to softer stools consistency. In addition, they observed a significant increase in Bifidobaterium among 0.8 g/ dL SYN-supplemented infants as compared to the control group, while the 0.4 g/ dL SYN-supplemented did not (129).

There is a concern that a possible harmful effect by adding prebiotics in infant formulas may be produced by the induction to more watery stools, which could increase the risk of dehydration in some infants, as pointed out by the European Society for Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) (111;138). The scientific committee on food of the European Commission reported that there is no conclusive data about water balance in infants fed with a prebiotic supplemented formula (138). Although previous studies assessed the effects of SYN1 on newborns (129), the study period was only four weeks, and the water balance was not assessed. Therefore, to demonstrate the safety and efficacy of the SYN1 supplementation in infants, there is still the need to assess the effect of SYN1 during a longer period, with special attention on water balance (for the potential risk of increasing watery stools and consequent dehydration).

HYPOTHESIS

2. HIPOTHESIS

Addition of SYN1 to infant formulae is safe and well tolerated by healthy neonates allowing appropriate growth and water balance. Furthermore, infants fed with the SYN1-formula have better composition of microflora and the stools characteristics and intestinal function are more closely to breastfed infants than infants fed with a standard formula.

OBJECTIVES

3. OBJECTIVES

Main objective

Demonstrate the tolerance, safety, and efficacy of a SYN1-supplemented infant formula (0.8g of Orafti® Synergy1/100 ml reconstituted milk) during the first 4 months of life.

Specific objectives

To demonstrate that during the first 4 months of life SYN1-supplemented formula is efficacy, safe and equally well tolerated as standard infant formula.

Tolerance:

- To evaluate the occurrence of digestive symptoms such as colics or regurgitation in SYN1 supplemented infants during the first four months of life, compared to non-supplemented infants.
- To analyze if there are differences in the quantity of formula ingested between the supplemented or non-supplemented group.

Safety:

- To analyze whether there are differences in growth among infants fed with the supplemented formula SYN1 and the infants fed with control formula during the first four months of life.
- To analyze if there are differences in the water-electrolyte balance, proteins, minerals and kidney function in infants fed with the supplemented formula or placebo formula, during the first three months of life.

Efficacy:

- To analyze whether infants with supplemented formula SYN1 improve bowel function by increasing stools frequency and favour softer stools (compared with infants fed with control formula).
- To analyze the efficiency of the formula-SYN1 in modulating the intestinal microflora composition, compared with un-supplemented control formula and breastfeeding
- To assess the influence of the SYN1 formula in the immune system and risk of allergy compared with infants fed with control formula.

METHODS

4. METHODS

4.1. STUDY DESIGN AND PLAN

Study design

Double-blind, randomized, placebo-controlled and parallel trial with 2 groups of neonates receiving an infant formula with or without (control) SYN1-supplementation (0.8 g of Beneo® Synergy1/100 ml) for 4 months. In parallel, a reference group of breastfed infants was recruited and followed for comparison to indicate the gold standard reference, as recommended by the Committee on Nutrition of ESPGHAN (139).

Study plan

All infants were recruited and allocated in a feeding group during the first month of life, and were followed up to the age of 4 months. One monthly visit to the hospital for examination of the infant and for the collection of samples, and of medical and nutritional data was planned, as well as phone calls in-between the visits during all the study progress to assess compliance. Table 6 shows the time schedule for the conduct of the study trial.

Table 6. Time schedule: schema of the study trial

	V0	C1	V1	C2	V2	C3	V3	C4	V4
Age (M)	**At birth**	**0.5**	**1**	**1.5**	**2**	**2.5**	**3**	**3.5**	**4**
Personal interviews/study visits									
Explanation of the study to parents	X								
Informed consent	X								
Randomisation	X								
Baseline data collection	X								
Sociodemographic data	X								
Visit: Anthropometry			X		X		X		X
Visit: medical history			X		X		X		X
Blood sample drawing (optional)							X		
Phone interviews									
Phone calls		X		X		X		X	
Parents at home (before coming to the visit)									
Fill in case report (4w		X		X		X		X	
Fill in 2-days diary (dietary intake and digestive			X		X		X		X
Food habits questionnaire			X		X		X		X
Stools sample collection	X		X				X		X
Urine sample collection			X				X		
End of the assessment									X

V: visit, C: call phone, M: months.

4.2. STUDY POPULATION

All infants were recruited at Hospital Universitari de Tarragona Joan XXIII and Hospital Universitari Sant Joan de Reus, mainly during their hospital stay for the birth or during the four following weeks (by phone call).

The inclusion criteria were:

- ✓ Healthy term infants (37 to 42 completed weeks of gestation).
- ✓ Normal birth weight (between 3rd and 97th percentiles according to Carrascosa Spanish graphs for term birth) (140).
- ✓ Age at recruitment < 4 weeks of life.
- ✓ Normal feeding behaviour or skills.

Specifically for formula fed infants:

- ≥90 % of energy intake fed by infant formula. There was no control of the infant formula or human milk consumed prior to enrolment.

Specifically for breastfed infants:

- ≥90 % of total milk volume fed by human milk (considering standard breast milk intake volumes, those published by the DARLING study) (141). There was no control of the infant formula or human milk consumed prior to enrolment.
- No complications in breastfeeding before hospital discharge (first 2-5 days of life).

- Mother's firm conviction to exclusively breastfed her infant during at least the first 4 months of life.

Breastfed infants who were included in the study from birth, but switched to exclusive infant formula during the first 4 weeks of life had the possibility to be randomized and allocated in 1 of the 2 study formula groups.

The exclusion criteria for recruitment were:

- ✓ Antibiotic treatment.
- ✓ Serious respiratory, neurological, gastrointestinal or metabolic disorders.
- ✓ Infections or other serious diseases that could hinder growth.
- ✓ Parents or guardians who cannot be expected to comply with protocol.
- ✓ Families whose mother's have not command of Spanish or Catalan languages.

The exclusion criteria to discontinue the study were:

- Any oral antibiotic treatment.
- Switching to other formulas or complementary feeding for more than 10% of the total energy intake or for more than 3 consecutive days.

Before the research team approached the families, the investigators checked the chosen feeding type, to inform them properly. Breastfeeding is considered as the gold standard for infants and was encouraged and

supported during all the study period. No information about infant formula distribution (to infants in formula fed groups) was given to families of breastfeed infants to avoid any negative influence on breastfeeding decision. Breastfed infants, who were included in the study from birth but switched to exclusive formula feeding during the first 4 weeks of life, were then informed and had the possibility to be randomized and allocated in 1 of the 2 study formula groups. Mothers who have previously decided to feed their infants with formula were approached and invited to participate in the randomized part of the study.

4.3. ADMINISTRATION OF PRODUCT TESTS

Study products description

Both infant formulas (control and SYN1-formula) were prepared specially for this trial. The test products were produced under the responsibility of BENEO-Orafti with a general composition matching current EU standards (European Law 2006/141/EU). Table 7 shows nutritional composition of SYN1-supplemented and control formulas. Both infant formulas contained the same amounts of lactose, protein, fat and micronutrients. The control formula was supplemented with an amount of maltodextrin (which is not expected to have any effect on the gut) equivalent to the weight of the SYN1 in the SYN1-supplemented formula. The test substance was SYN1 (0.8 g/dl), a chicory-derived fructan formulation with an active fructan that is composed of approximately 50% oligofructose (degree of polymerization [DP] <10) and 50% long-chain inulin (DP ≥10).

Treatment administration

The infant formulas used were provided to the volunteers for free during their participation in the study and after until they changed to follow-on formula (as indicated by their paediatricians). The infant formulas were administered to the infant for every meal during the first 4 months of life. The subjects should not alter their usual feeding regimen during the intervention period.

Participants received instructions to prepare the infant formula as standardized. The instructions were that the bottle should be prepared adding a scoopful of 4.3g (14.33g/100 ml, 14.33% solution) powdered infant formula for each 30mL of water. First, the water should be put in the bottle and second the powdered formula. After that, the bottle should be shaken to favour the dilution.

The group of breastfed infants in the study received free diapers during the 4 months that lasted the follow-up.

Table 7. Nutrients composition per 100ml of study infant formulas.

Nutrient	Units	Control formula	Synergy1 formula
Energy value	kcal	66,8	64,9
Proteins	g	1,5	1,5
Whey protein	g	0,9	0,9
Casein (40%)	g	0,6	0,6
Fat	g	3,6	3,6
Carbohydrates	g	7,2	6,4
Lactose	g	6,3	6,3
Maltodextrin	g	0,9	0,1
Fibre (SYN1)	g	-	0,83
Minerals			

Sodium	mg	32,3	32,3
Potassium	mg	82,6	82,6
Chloride	mg	45,2	45,2
Calcium	mg	63,2	63,2
Phosphorous	mg	42,6	42,6
Iron	mg	0,6	0,6
Magnesium	mg	7,2	7,2
Zinc	mg	0,6	0,6
Copper	mg	38,1	38,1
Iodine	mcg	9,7	9,7
Manganese	mcg	10,3	10,3
Selenium	mcg	6,6	6,6
Vitamins			
Vitamin A	mcg	66	66
Vitamin D	mcg	1,1	1,1
Vitamin E	mg	0,8	0,8
Vitamin K	mcg	5,8	5,8
Vitamin B1	mcg	79	79
Vitamin B2*	mcg	168	168
Vitamin B6	mcg	48	48
Vitamin B12*	mcg	0	0
Vitamin C	mg	12	12
Folic Acid	mcg	12	12
Calcium Panth.	mg	0,5	0,5
Nicotinamide	mg	0,6	0,6
Biotine	mcg	3,2	3,2
Others			
Choline	mg	9,7	9,7
Taurine	mg	2,6	2,6
Inositol	mg	3,4	3,4
L-Carnitine	mg	1,9	1,9

Blinding, randomization and allocation concealment

Four different infant formula containers were created (identified only by different colours: yellow, red, blue and green), two for each study formula (to better protect the blinding of the intervention). The infant formulas were packed in sealed boxes labelled in such a way that the content of SYN1 and maltodextrin were unknown to both the study investigators and the parents of the study subjects. The code (correspondence between colour and treatment) was blinded to all of the investigators, participants and caregivers during all the study progress (until the end of the study after all data were introduced in the data base and statistically processed) and was not blinded to manufacturer.

The random list of treatments was prepared by an investigator (not involved in recruiting families and assigning treatments) using the computer program EPIDAT (Servizo de Epidemioloxía da Dirección Xeral de Innovación e Xestión da Saúde Pública da Consellería de Sanidad).

Allocation concealment was ensured by sequentially numbered sealed envelopes not accessible to investigators until the informed consent was signed. The envelopes were closed and contained the name of a treatment that could be: yellow, red, green or blue and a randomization number.

Compliance *(phone calls)*

Families' adherence to the treatment was checked every two weeks alternatively by phone calls or during study visits. During the phone or personal interviews, the investigators asked the families, if they had included any other infant formula, supplement or complementary feeding.

4.4. MONITORING AND HANDLING OF ADVERSE EVENTS

Collection of information about adverse events:

1. Families were encouraged (and provided with mobile phone numbers) to contact the study team if they felt that the infant formula had any adverse effect on the infant (like vomiting, regurgitation, diarrhoea, colics, excessive crying, etc)
2. The study team asked monthly by telephone interviews to the mothers how they felt the study formula was accepted by their infants.
3. The families recorded in a monthly 2-days diary any vomit, regurgitation and number and consistency of depositions that the infants made.
4. The families were asked at the visits about their feeling on infant's digestive comfort/discomfort and any illness and medication.
5. All the withdrawals were recorded in a data base during the study progress with the reason to withdraw and the type of study formula.
6. Approximately, every 3 months, the study team tested the reasons to withdraw for each study formula "colour mark".

The adverse events detected by the research team were individually recorded in specific forms (Appendix 9.1). After completing the adverse event form by infant, the events were classified.

Classification of adverse events:

- Serious adverse event

A serious adverse event is any untoward medical occurrence that at any dose results in death, is life-threatening at the time of the event, requires inpatient hospitalization or is another important medical event. The following cases are examples of serious adverse events:

- Severe malabsorption
- Dehydration
- Metabolic acidosis

- Non-serious adverse events

- Digestive discomfort
    - Vomiting and gastroesophagical reflux
    - Loose stools
    - Colics (cramps, belly pain)
    - Parents' report that the infant "doesn't tolerate the formula"
- Cow's milk intolerance suspicion (skin rash, bloody stools)
- Lack of weight gain

Intensity:

The intensity grades of a non-serious adverse event were rated by the study paediatricians into intense, moderate or slight. In a general approach, any non-serious adverse event that did not induce to withdraw was considered as a slight adverse event.

Causality assessment:

In order to detect any potential adverse event related to the study product during the study progress, all adverse events and digestive symptoms (even if not expected to be related to the study formula) were recorded in a data base and classified by type of colour of infant formula. It was recorded if the adverse event disappeared with the treatment abandonment and if it appeared again after the reintroduction.

If any of the containers had shown significantly higher rates of secondary effects, and infants would run any health risk, the team would have been able to consider the unblinding of the study products or the end of the trial.

The study would have been stopped if:

- any death potentially due to the study formula
- three or more serious adverse events potentially due to the study formula
- repeated moderate adverse events potentially due to the study formula

4.5. ASSESSMENT PROCEDURES

The follow table (Table 8) shows the list of outcomes by collection method at any timepoint of the study follow-up.

Table 8. List of outcomes by collection method and the timepoint.

Outcomes	Method	Timepoint
Baseline data		
Initials, date of birth, gender, gestational age, APGAR score	MR	V0
Antibiotic intake by mother during 4 weeks prior to delivery	MR	
Antibiotic treatment of the infant at birth	MR	
Perinatal data: anthropometrical data (weight, length and head circumference), type of delivery (vaginal or caesarean)	MR	
Family medical history/course of pregnancy	PI	
Socio-demographic data	PI	
Anthropometry		
Weight, length, head circumference, waist circumference, mid upper arm circumference, Tricipital skin fold, Sub scapular skin fold	IA	V1, V2, V3 & V4
Questionnaires	filled in/or collected	
a) Medical history/Digestive Symptom questionnaire (recall last 4 weeks): Stools frequency and consistency, digestive discomfort, vomiting, regurgitation, illness events, medication of infants or breastfed mothers, allergy (atopic dermatitis, atopic family and history vaccination	PI	V1, V2, V3 & V4
b) Infant's behaviour questionnaire: crying behaviour (recall 4 weeks)	PI	V1, V2, V3 V4
c) Telephone interview checklist: to monitor compliance, acceptance of formula, and potential supplementation	PI	T1, T2, T3, T4
Parents Diary	collected	
a) Dietary intake: 2-days food diary the two days before	PR	V1, V2, V3

Outcomes	Method	Timepoint
visits		& V4
b) Digestive symptoms: 2-days diary records on vomiting, regurgitation, frequency and consistency of depositions to be filled in at the two days before visits	PR	V1, V2, V3 & V4
c) Case report form: to be filled in just in case of illness events, vaccination and medication during periods between visits	PR	V1, V2, V3 & V4
d) Feeding habits and behaviour: infants' appetite and crying behaviour to be filled in the day before visits	PR	V1, V2, V3 & V4
Laboratory analysis		
a) Urine: Osmolarity, Na, K, Cl, creatinine	PC/ LAB	V1 & V3
b) Stools: Microbial composition (bacteroides, bifidobacteria, clostridium coccoides, clostridium leptum, enterobacteriacea, total counts of bacteria)	PC/ LAB	V0 & V3
c) Blood: pH; minerals: Na, K, Cl; cholesterol; total protein, pre-albumin, albumin, urea, high sensibility CRP, antibody titres to vaccinations as performed and Immunoglobulin (Difteria IgG, Tetanos IgG, Pertusis IgG, total IgG, IgE and IgM)	LAB	V3

MR: medical records at hospital; PI: parents' interview by investigators; IA: investigator assessment; PR: parents' report; PC: parents collection; LAB: laboratory analyses.

4.5.1. Data collection methods

Baseline data

Baseline data was collected from medical records during the hospital stay.

Anthropometry

The nude weight and length of the infant was determined with a SECA 336 baby scale (precision: ±10g) and a SECA 232 stadiometer (precision:

±1mm), respectively. To measure skinfold thicknesses, a Holtain calliper was used (precision: ±0.2mm) and for the rest of measurements a non-extendable insertion tape was used (precision: ±1mm). The devices were monthly calibrated. Measurements were taken by trained personnel, following the WHO recommendations based on the Lohman reference manual (142).

Measurements were taken in duplicate, except skinfold thickness, which were taken in triplicate. To minimize inter-observer variability, skinfold thickness measurement procedures were carefully standardized. The tricipital skinfold was measured in the posterior midline of the left arm, over the triceps muscle, at the midway point between the lateral projection of the acromion process of the scapula and the inferior margin of the olecranon process of the ulna, with the arm hanging loosely and comfortably at the subject's side. The subscapular skinfold, on the left side, just below to the inferior angle of the scapula. The skinfold was picked up on a diagonal, inclined infero-laterally at approximately 45° vs. the horizontal plane in the natural cleavage lines of the skin. Once located, measurement sites were marked with a pen. The thumb and index finger of the observer were used to elevate a double fold of skin and subcutaneous adipose tissue about 1 cm proximal to the site at which the skinfold was measured. The measurements were repeated and recorded three times after excluding any clearly erroneous value (>5%).

Questionnaires

a) Medical History/Digestive symptom questionnaire.

During the visits, the research team interviewed parents about digestive functioning, illnesses, etc. during the previous month (Appendix 9.2).

b) Infant's Behaviour Questionnaire

During the visits, the researcher team interviewed parents about the crying behaviour of their infant during the previous month (Appendix 9.2).

c) Telephone interview checklist

Telephone interviews for compliance check were performed 2 weeks after visits by the study personnel. Parents were asked for feeding type, acceptance of formula, and potential supplementation of infants. Parents were reminded to complete the diary, to come to next visit, to collect samples (urine/faces) and to vaccinated infants.

Parents Diary

a) Dietary intake

A diary was filled in by the parents the two subsequent days just before the visits. The diary form requested information on formula intake (volume) in each meal during the 2 days prior to the visit. During the visit, nutritionists checked if the diaries were filled in correctly asking the parents about the preparation of the formula and checking total volumes reported by parents.

b) Digestive symptoms

A diary was filled in by the parents the two subsequent days just before the visits. The diary form requested information on tolerance (vomiting and regurgitation), stools frequency and stools consistency (Bristol Scale score) (143) (Appendix 9.2).

c) Case report form

This form was filled in by parents at home, during all time between visits (from V0 to V1, from V1 to V2, from V2 to V3 and from V3 to V4). Parents were asked to write down all vaccinations and all illness events and medication during periods between visits.

d) Feeding habits and behaviour

A questionnaire on infants' appetite and crying behaviour was filled in by parents at home the day just before the visits (Appendix 9.2).

Laboratory Analysis

a) Urine collection

Parents were provided with:

- Written instructions.
- Plastic bags to be put in the diaper.
- Sterile vials to storage the samples.
- Temperature preservation bags.
- Icy patches to put into the preservation bags.

Parents should put the plastic bag on the diaper. The diaper should be checked each 5-10 minutes and the plastic bag changed each 30 minutes until the sample was collected.

The urine analyses were performed in only 1 central laboratory (Laboratori Hospital Universitari de Tarragona Joan XXIII, Institut Català de la Salut).
The samples picked in the Hospital Sant Joan de Reus were processed and stored at -20ºC until its transport (within 1 week) and analysis in the centralized laboratory. Urinary Cl, K and Na were determined by indirect

potenciometry and creatinine by Jaffe reaction method in a Siemens ADVIA 2400 (Siemens Medical Solutions and Diagnostics, Dublin, Ireland) using standardized protocols; osmolarity was measured by the USC method in a ARKAY Osmo Station OM-6050 (A. Menarini Diagnostics, Valkenswaard, Nederland).

b) Stools

Parents were provided with:

- Oral instructions.
- Sterile vials to storage the samples (which contained plastic spoons to transfer the stools samples from the plastic bag to the sterile vial).
- Temperature preservation bags.
- Icy patches to put into the preservation bags.

Stools samples were collected at birth, 1st, 3rd and 4th month (±5 days) of life and were brought frozen to the study unit at the following visits. If the baby defecated during the visit, an additional sample was taken, and used instead of the sample collected at home.

An amount > 2g of stools was filled in the sterile vial using the plastic spoon, to be frozen as soon as possible at -20°C for later analysis. At least 2g of stools were necessary for analysis. Bacterial flora analysis of the stools samples for Bifidobacterium, Bacteroides, Enterobacteriaceae and total bacteria was done by qPRC technique, in a subgroup of infants (approximately 50 per group) in the samples from baseline and month 3. Stools were stored at the Hospitals (for maximum of 6 months) and were

then sent to a central laboratory (Institut für Mikroökologie, Auf den Lüppen 8, Herboron, Germany) to start the procedure.

DNA was extracted using Easy Mag DNA Isolation system (BioMerieux, Nuertingen, Germany) according to the manufacturer's instructions. Quantitative PCR amplification and detection were carried out using the primers. PCR amplification and detection was performed using an ABI 7300 Real Time PCR System (Applied Biosystems, Darmstadt, Germany) in optical-grade 96-well plates sealed with the optical sealing tape. Each reaction mixture (25 µl) comprised 12.5 µl of QuantiTect SYBR Green PCR Master Mix (Qiagen, Hilden, Germany), 1,6 µl of primer mixes (10 mol/µl each), 9,4µl of sterile distilled water, and 1.5 µl of stool DNA (10 ng/µl). For the negative control, 1,5 µl of sterile distilled water instead of the template DNA solution was added to the reaction solution. A standard curve was produced using the appropriate reference organism to quantify the qPCR values into numbers of bacteria per gram stool. The standard curves were prepared using the same PCR assays as for the samples. The fluorescent products were detected in the last step of each cycle. A melting curve analysis was carried out after amplification to distinguish the targeted PCR products from the non-targeted PCR products. The melting curves were obtained by slow heating at temperatures of 60° C to 95° C at a rate of 0.2° C/second, with continuous fluorescence collection. The data was analyzed using the ABI 7300 Real Time PCR System Sequence Detection Software Version 1.4. The real-time PCRs were performed in triplicate, and average values were used for enumeration. The amplification program used consisted of one cycle of 95° C for 15 minutes and then 40 cycles of 95° C for 30 seconds, 50 to 60 °C for 60

seconds(depending on primers, see table 1 annealing temperatures). The detection limit was 10^5 cfu/g of wet feces.

If oral antibiotic treatment was reported (either the infant or the mother of a breastfed infant) or the child started complementary feeding (>10 % of total energy intake), the faecal sample was not analyzed and the infant was excluded from per protocol analyses.

c) Blood analysis

Parents brought their infants to the hospital and a nurse collected the blood sample. The amount of whole blood collected was 3.5 mL. Ions equilibrium was analysed immediately at the laboratory of each Hospital, and the rest of blood parameters were analyzed at only 1 central laboratory (Laboratori Hospital Universitari de Tarragona Joan XXIII, Institut Català de la Salut).

The samples picked in the Hospital Sant Joan de Reus were processed and stored at 4ºC until its transport (within the same morning) and analysis in the centralized laboratory. Cl, K and Na were determined by indirect potentiometry, total proteins by Biuret method, albumin by bromocresol purple method; C reactive protein by a turbidimetric method and urea and cholesterol by a enzymatic method in a Siemens ADVIA 2400 (Siemens Medical Solutions and Diagnostics, Dublin, Ireland) using standardized protocols; prealbumin, Ig M, Ig G and Ig E were analyzed by nephelometry in a Siemens BN II (Siemens Medical Solutions and Diagnostics, Dublin, Ireland); Ig G Tetanus and IgG Diphteria were measured by enzimoimmunoanalysis in a Genzyme VIROTECH Minilyser (Genzyme VIROTECH GmbH, Russelsheim, Denmarky) and a manual ELISA (Serion

Immundiagnostica GmbH, Würzburg, Germany) respectively; Ig G Pertussis was measured by indirect immunofluorescence using antibodies from Bios GmbH (BIOS GmbH, Munich, Germany).

4.6. STATISTICAL

4.6.1. Sample size calculation

The sample size (64 per group) was calculated to allow detection of a difference of 0.5 SD in weight gain (as the main safety outcome measure) over the study period. In order to terminate the study with 64 infants in each group, 100 breastfed infants (BF) and 200 formula fed infants (FF; 100 in each formula group) were planned to be recruited. During the field work, we envisaged a dropout rate higher than the forecasted (35%). Therefore in order to not lose statistical power we randomized 52 additional participants after the first 200 formula-fed infants had been recruited. Only the formula-fed (randomized) groups were included in the per protocol analyses. It was planned to extract blood samples only in a subsample of 30 infants/ feeding group and to perform microflora analysis in a subsample of 50 infants per feeding group.

4.6.2. Data management

Personal data to allow contact study participants (name, address, telephone) were recorded in a data base only available under password for investigators directly related to the project. This personal data information was always separated from all the collected data and form sheets.

All infants were identified at form sheets and faecal samples by initials, a screening number and a randomization number. The screening number was a correlative number given to the infant by order of inclusion in the

study. The randomization number was a correlative number as well, given only to formula fed infants by order, according to the treatment list, associated to a treatment.

A Microsoft Access® data base to introduce all collected data was designed and placed in a server only available under password for investigators. Infants were as well identified by initials, screening and randomization numbers.

4.6.3. Analyses

Tables 9 and 10 show all the outcome variables that were analysed.

Table 9. List of continuous variables.

Continuous variables	Units
Anthropometry	
Birth weight (g)	g
Birth length (cm)	cm
Birth head circumference (cm)	cm
Gestational age	Complete weeks
Weight at months 1, 2, 3 & 4	g
Length at months 1, 2, 3 & 4	cm
Head circumference at months 1, 2, 3 & 4	cm
Waist circumference at months 1, 2, 3 & 4	cm
Arm circumference at months 1, 2, 3 & 4	cm
Tricipital skinfold at months 1, 2, 3 & 4	mm
Subscapular skinfold at months 1, 2, 3 & 4	mm
Formula intake	
Average intake at months 1, 2, 3 & 4	ml
Average intake/weight at months 1, 2, 3 & 4	ml/kg

Continuous variables	Units
Average energy intake/weight at month 1, 2, 3 & 4	Kcal/kg
Urinary laboratory parameters	
Cl at month 1 & 3	mEq/L
K at month 1 & 3	mEq/L
Na at month 1 & 3	mEq/L
Na/K ratio at month 1 & 3	Ratio
Creatinine at month 1 & 3	mEq/L
Osmolarity at month 1 & 3	mOsm/L
Serum laboratory parameters	
Cholesterol at month 3	mg/dL
Urea at month 3	mg/dL
Albumin at month 3	g/dL
Proteins at month 3	g/dL
Na at month 3	mEq/L
K at month 3	mEq/L
Cl at month 3	mEq/L
Prealbumin at month 3	mg/dL
pH at month 3	
Base excess at month 3	mmol/L
Ca_ion at month 3	mmol/L
IgG at month 3	mg/dL
IgM at month 3	mg/dL
IgE at month 3	UI/mL
IgG Diphteria at month 3	UI/mL
IgG Tetanus at month 3	UI/mL
Digestive symptoms	
Depositions at months 1, 2, 3 & 4	n/day
Regurgitation at months 1, 2, 3 & 4	n/day
Vomits at months 1, 2, 3 & 4	n/day
Faecal consistency score (Bristol scale) at months 1, 2, 3 & 4	Score (1 to 7)
Infant's infection	

Continuous variables	Units
Infant's infection episodes at month 1, 2, 3 & 4	n
Infant's non-infection episodes at month 1, 2, 3 & 4	n
Child's behaviour	
Crying episodes	n/day
Crying time	min/day
Microbiota faecal samples	
Bacteroides	log cfu/g faeces
Bifidobacteria	log cfu /g faeces
C. coccoides	log cfu /g faeces
C. leptum	log cfu /g faeces
Entero-bacteriaceae	log cfu /g faeces
Total counts	log cfu /g faeces

Table 10. List of string variables.

String variables	Categories
Sociodemographic	
Gender	Male/female
Delivery way	Vaginal/caesarean section
Mother's nationality	Foreigner/Spanish
Father's nationality	Foreigner/Spanish
Mother working the previous week	No/yes
Father working the previous week	No/yes
Mother's occupation level	Low/medium/high
Father's occupation level	Low/medium/high
Mother's education level	ISCED 0, 1, 2, 3, 4, 5 & 6
Father's education level	ISCED 0, 1, 2, 3, 4, 5 & 6
Serum immune parameters	
Serum CRP (over detection limit)	No/yes
IgG Diphteria cat (over detection limit)	No/yes
IgE response (over detection limit)	No/yes

String variables	Categories
IgG Pertusis (over detection limit)	No/yes
Study formula acceptance	
Infants acceptance at month 1, 2, 3 & 4	Very good, good, moderate, poor & very
Frequency of digestive symptoms	
Frequent digestive discomfort at month 1, 2, 3 & 4	No/yes
Frequent vomits at month 1, 2, 3 & 4	No/yes
Frequent regurgitation at month 1, 2, 3 & 4	No/yes
Atopic dermatitis	
Presence of atopic dermatitis at month 1, 2, 3 & 4	No/yes

Cfu: colony-forming unit; ISCED: International Standard Classification of Education - 1997 version, UNESCO Institute for Statistics 2006 Pre-edition. ISCED 0: Preliminary level of education, ISCED 1: Primary level of education, ISCED 2: Lower secondary level of education, ISCED 3: Upper secondary level of education, ISCED 4: Postsecondary nontertiary, ISCED 5: First stage of tertiary education, ISCED 6: Second stage of tertiary education.

The descriptive results were expressed as means (±SD) or medians and interquartile ranges (IQR) for normal and skewed variables respectively, after assessing the normal distribution of variables with a Kolmogorov-Smirnov test.

Categorical variables are presented as absolute and relative frequencies (n and %, respectively). The faecal microbiota concentrations were converted to base-10 logarithms and are presented as absolute cell counts and as % of total bacteria (calculated with non-transformed data).

T-tests or Mann-Whitney U-tests were used for statistical comparisons during the cross-sectional analyses between feeding groups, as requested by normally distributed or none normally distributed variables. Pearson chi-square and Fisher's exact tests (if observed frequencies were under

expected frequencies) were used for statistical comparison of categorical data.

Longitudinal changes were analyzed in normal distributed variables using a T-Test for repeated measures (for each feeding). For skewed variables, Wilcoxon tests for repeated measurements were applied for 2 group comparisons. Mean longitudinal changes for parameters assessed more than twice, i.e. growth, deposition number or faecal consistency score were tested by applying a one-way linear model for repeated measures.

A linear regression analysis including formula type and bacterial counts was performed to show the effects on faecal consistency and frequency.

A Cox regression was applied to compare withdrawals rates between study groups during the whole study period.

In addition, we performed intention-to-treat analyses to test for differences between feeding groups as well.

The statistical significance was accepted at $p<0.05$. The data management and statistical analyses were conducted using the IBM SPSS version 20.0 (IBM Corp., Armonk, NY, USA).

4.7. ETHICAL CONDUCT OF THE CLINICAL TRIAL

The study has been conducted following the Helsinki declaration (144) and guidelines for ethical conduct of medical research involving children (145).

The protocol and the study conducted (enrolment, intervention allocation, follow-up, control of adverse events and data analysis) was designed following the CONSORT Guidelines for clinical trials (146;147).
The study protocol was submitted and approved by the Ethical Committees of both Hospitals where the study was conducted.

Written informed consent was obtained from each participant by the investigator prior to the enrolment in the study. The consent form was signed and dated by the father or the mother or the legal representative of the infant (Appendix 9.3).

The study was registered as Study on Fermentable Carbohydrates in Healthy Infants (BAMBINOL) (NCT00808756) at clinicaltrials.gov.

4.8. QUALITY CONTROL AND MONITORING

Recruitment and follow-up

Nutritionists in charge of the recruitment were trained to provide families all the information and to collect baseline information and faecal samples after obtaining written consent.

Nutritionists were in close contact with participating families during all the study period to ensure adherence to treatment and answering all possible doubts that could rise among families. To enhance communication with the research team, families were provided with the nutritionist's mobile phone numbers. So that, participants could contact the team as soon as they needed to be provided with formula or if they had any doubt in relation to questionnaires or visits.

Dietary intake assessment

Nutritionists were trained to revise food diaries of participants to obtain the most accurate information during the study visits. So that, they check preparation of formula dilution, final volumes offered and final volumes consumed (148) and then re-checked with families the possible inclusion of other ingredients or supplements that could influence any of the outcomes (as laxatives, cereals, fruits, fruit juice, etc).

Anthropometry

Training: nutritionists conducting the study trial were provided with Standard Operating Procedures (SOPs), based on the World Health Standards (142;149;150). They were trained by senior scientists, expert in assessing infant growth and doing anthropometrical measurements during

the previous month to the beginning of the study. Furthermore, the team performed repeated measures to the same infants to ensure harmonization of measures prior to the study beginning and during the conduct of the study trial.

Repeated measures: to ensure precision of anthropometrical measurements, all measures were taken in duplicate unless skinfolds, which were taken in triplicate. Differences in duplicate or triplicate measures higher than the 5% were considered poorly precise and repeated.

Calibration of material: the baby precision scales were calibrated using certified 5Kg (nb. Z041612) and 10Kg (nb. Z06073787) Cofrac Class M1 (1mg-20Kg) standard weights for test points 5, 10 and 15Kg. The infant measuring tables were calibrated by using metal test rods of 100 and 600mm. Precisions testing gauge for Holtain calliper was used to test the points 10, 20, 30 and 40 mm.

Blinding of the study formulas

As previously commented, we performed a random list using EPIDAT computer program. After, investigators of the unit not directly involved in recruitment, introduced the treatments by order into previously numbered envelopes. So that the responsible investigators of recruiting infants only should take the envelopes by order at arrival of participants, remaining completely blinded.

The investigators, the laboratory analyses and the families were all blinded during all the study.

The treatment was not revealed to investigators until the follow up of all infants enrolled in the study was completed and all the data was introduced in the data base.

Microbiological analyses of infant formulas

The real-time PCRs were performed in triplicate, and average values were used for enumeration.

Laboratory quality certificates

Both of the laboratories in charge of analyzing urine and blood samples were certified with the ISO9001:2000.

Faecal and urinary samples preservation and transport

Families transport: to ensure proper transport of urine and faecal samples to the study centres, isolating bags and ice patches were provided to all families. So that, urine samples arrived refrigerated to study centres and faecal samples remained in freezing conditions during its transport the study centre.

Preservation at Biobancs: faecal samples were preserved in monitored freezers (-20ºC) in Biobancs at local study centres, until its shipment to the central laboratory.

Transport to Final Laboratory: samples were sent from local Biobancs to the laboratory in Germany (in 3 shipments through all the study period) in dry ice in maximum 48 to 72h, ensuring correct preservation of stools for further analyses.

RESULTS

5. RESULTS

Some results of this thesis had been previously published in a peer review journal (appendix 9.4).

5.1. BASELINE DATA

5.1.1. Baseline characteristics

Infants allocated in the different feeding groups were similar. Genders were distributed similarly in all feeding groups at recruitment (Table 11). Within the control group there were a higher proportion of caesarean section deliveries than the SYN1 group (32.3% vs. 18.8% in control and SYN1 respectively, $p<0.05$) (Table 11).

Table 11. Distribution of gender and delivery type within feeding groups (all recruited infants).

	Control N (%)	SYN1 N (%)	Breastfed N (%)	Control vs. SYN1	Breastfed vs. FF
Gender					
Male	67 (54)	62 (48.4)	66 (48.5)	0.374	0.617
Female	57 (46)	66 (51.6)	70 (51.5)		
Delivery way					
Vaginal	84 (67.7)	104 (81.3)	104(76.5)	**0.014**	0.684
Caesarean	40 (32.3)	24 (18.8)	32 (23.5)		

FF: formula fed.

There was no difference between formula groups (control & SYN1) or between infants fed formula and breastfed in any on the anthropometrical variables or gestational age at birth (Table 12).

Table 12. Anthropometrical characteristics at birth of all infants recruited by feeding groups.

	Control Mean (SD)	SYN1 Mean (SD)	Breastfed Mean (SD)	Control Vs. SYN1
	(n = 124)	(n = 128)	(n = 131)	
Birth Weight (g)	3235 (346)	3289 (385)	3302 (346)	0.248
Birth Length (cm)	49.5 (2.0)	49.7 (1.8)	49.8 (1.6)	0.376
Birth Head circunference (cm)	34.2 (1.3)	34.3 (1.2)	34.5 (1.2)	0.600
Gestational age (weeks)	39.9 (1.3)	39.7 (1.1)	39.9 (1.3)	0.394

*No significant differences among control formula, SYN1 and breastfed group.

5.1.2. Sociodemographic characteristics

Sociodemographic characteristics of the family, like parents' nationality, working status, type of occupation or education level were similar in both formula groups (Tables 13 and 14).

As reported previously in the literature, breastfed infants differed from formula fed infants in many socioeconomic parameters. Mothers and fathers of infants who were breastfed had higher proportion of qualified work positions (Table 13) as well as highest education levels (Table 14). Mother's nationality differed between formula fed and breastfed infants, as well; having a higher proportion of foreign mothers those infants who were breastfed (4.1% vs. 0.42%).

Table 13. Socioeconomic characteristics by feeding group.

	Control N (%)	SYN1 N (%)	Breastfed N (%)	Control vs. SYN1	BF vs. FF
Mother's Nationality					
Foreigner	0 (0)	1 (0.83)	5 (4.07)	0.316	**0.010**
Spanish	120 (100)	119 (99.2)	118 (95.9)		
Father's Nationality					
Foreigner	9 (7.6)	6 (5.0)	8 (6.5)	0.424	0.941
Spanish	110 (92.4)	113 (95.0)	115 (93.5)		
Mother working the previous week					
No	54 (45.8)	56 (47.5)	31 (25.4)	0.794	**<0.001**
Yes	64 (54.2)	62 (52.5)	91 (74.6)		
Father working the previous week					
No	19 (16.1)	20 (17.5)	14 (11.45)	0.769	0.181
Yes	99 (83.9)	94 (82.5)	108 (88.5)		
Mother's occupation level					
Low qualified	17 (14.9)	18 (16.2)	7 (6.1)		
Medium qualified	81 (71.0)	82 (73.9)	62 (53.9)	0.631	**<0.001**
High qualified	16 (14.0)	11 (9.9)	46 (40.0)		
Father's occupation level					
Low qualified	61 (54.5)	52 (48.6)	26 (21.7)		
Medium qualified	33 (29.5)	41 (38.3)	48 (40.0)	0.374	**<0.001**
High qualified	18 (16.1)	14 (13.1)	46 (38.3)		

BF: breastfed, FF: formula fed.

This similar distribution of infants and their characteristics in the two formula groups did not change with withdrawals occurred during the whole study. These results show that equal randomization of infants was kept throughout the study.

Table 14. Distribution of parents in education level category, by feeding groups.

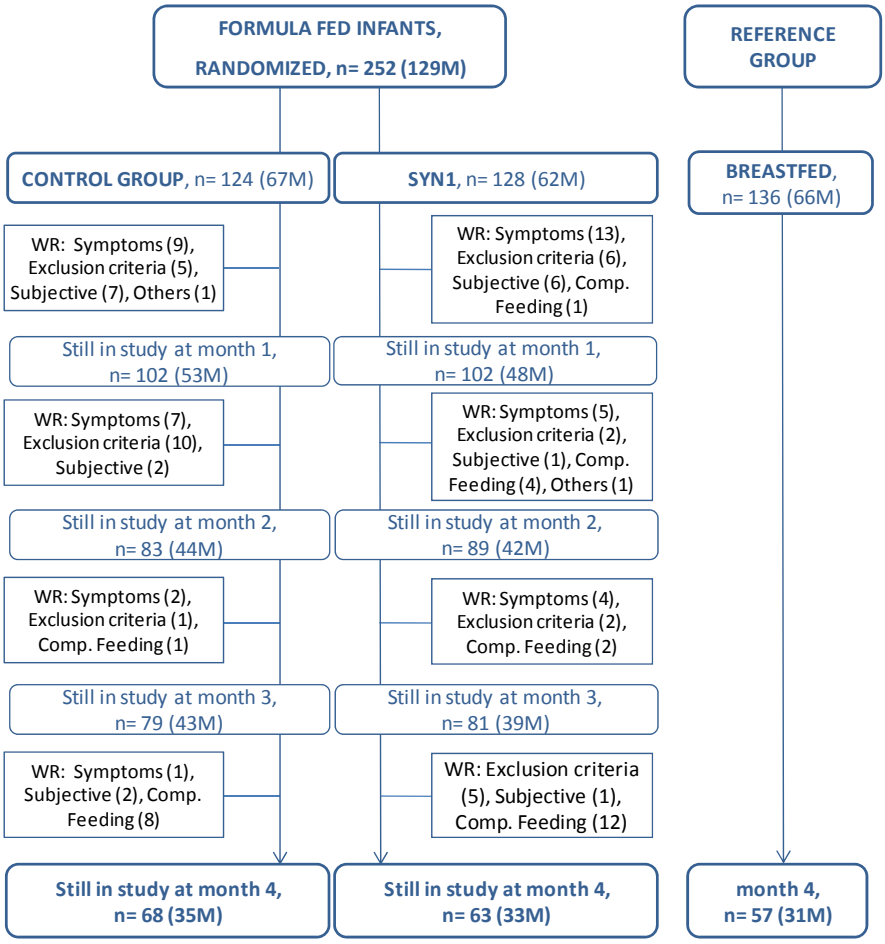
	Control N (%)	SYN1 N (%)	Breastfed N (%)	Control vs. SYN1	BF vs. FF
Mother's education level					
Preliminary level of education	1 (0.9)	0 (0)	0 (0)		
Primary level of education	11 (9.3)	9 (7.9)	4 (3.3)		
Lower secondary level of education	35 (29.7)	43 (37.7)	16 (13.2)		
Upper secondary level of education	36 (30.5)	25 (21.9)	20 (16.5)	0.550	**<0.001**
Post-secondary non-tertiary	12 (10.2)	13 (11.4)	23 (19.0)		
First stage of tertiary education	23 (19.5)	24 (21.1)	56 (46.3)		
Second stage of tertiary education	0 (0)	0 (0)	2 (1.7)		
Father's education level					
Preliminary level of education	0 (0)	0 (0)	1 (0.83)		
Primary level of education	18 (15.7)	17 (15.6)	9 (7.4)		
Lower secondary level of education	48 (41.7)	30 (27.5)	16 (13.2)		
Upper secondary level of education	26 (22.6)	27 (24.78)	27 (22.3)	0.069	**<0.001**
Post-secondary non-tertiary	15 (13.0)	14 (12.8)	26 (21.5)		
First stage of tertiary education	8 (7)	20 (18.4)	40 (33.1)		
Second stage of tertiary education	0 (0)	1 (0.9)	2 (1.7)		

BF: breastfed, FF: formula fed.

5.2. STUDY SAMPLE FOLLOW-UP AND WITHDRAWAL ANALYSES

Figure 10 shows the participants in each study timepoint.

Figure 10. Recruitment, randomization and follow-up and withdrawals at each study timepoint.



M: males, WR: withdrawal reason.

Cross-sectional analysis

Both the total number of withdrawals and the number of withdrawals per reason group were distributed similarly between formula groups during each of the 4 months. Dropouts/withdrawals at any timepoint did not affect the similar distribution of infants in both study formula groups. Neither the number of infants in each feeding group, nor the gender or any other of the baseline characteristics (see corresponding sections above) differed between feeding groups at any of the time points (month 1, 2, 3, 4). Table 15 and 16 show detailed information about all cases and reasons to dropout or withdraw from the study. Breastfed infants, as expected, presented different distribution of dropout reasons than formula fed infants. The reason for the high number of dropouts because of "exclusion criteria" in the breastfed group, especially in the first two months, was that breastfeeding, as diet alone, could not be maintained throughout the study duration.

Table 15. Detailed description of drop out reasons at each timepoint.

	Control N (%)	**SYN1** N (%)	**Breastfed** N (%)	**Control vs. SYN1**
MONTH 1				
Symptoms	9 (40.9)	13 (50)	1 (2)	
Vomiting & gastroesophagical reflux	5 (55.6)	4 (30.8)	0 (0)	
Loose stools	2 (22.2)	1 (7.7)	0 (0)	
Lactose or cow's milk intolerance suspicion	0 (0)	4 (30.8)	0 (0)	NS
Colic	1 (11.1)	1 (7.7)	0 (0)	
Lack of weight gain	1 (11.1)	2 (15.4)	1 (100)	
Constipation	0 (0)	1 (7.7)	0 (0)	

	Control N (%)	SYN1 N (%)	Breastfed N (%)	Control vs. SYN1
Exclusion criteria	5 (22.7)	6 (23.1)	20 (40.8)	
Antibiotics/ drugs that may influence flora	3 (60)	4 (66.7)	9 (45)	NS
Contact lost	2 (40)	2 (33.3)	11 (55)	
Subjective	7 (31.8)	6 (23.1)	-	
Mothers reporting non-tolerance	3 (42.9)	3 (50)	-	
Parents believe that study formula did not feed	2 (28.56)	1 (16.7)	-	NS
Lack of confidence	2 (28.6)	2 (33.3)	-	
Introduction complementary feeding	0 (0)	1 (3.8)	0 (0)	
Others	1 (4.6)	0 (0)	4 (8.2)	
Breastfeeding abandonment	-	-	24 (49)	
MONTH 2				
Symptoms	7 (36.8)	5 (38.5)	1 (12.5)	
Vomiting & gastroesophagical reflux	2 (28.6)	3 (60)	0 (0)	
Loose stools	2 (28.6)	0 (0)	0 (0)	
Lactose or cow's milk intolerance	1 (14.23)	2 (40)	0 (0)	NS
Colic	2 (28.6)	0 (0)	0 (0)	
Lack of weight gain	0 (0)	0 (0)	1 (100)	
Constipation	0 (0)	0 (0)	0 (0)	
Exclusion criteria	10 (52.6)	2 (15.4)	2 (25)	
Antibiotics/ drugs that may influence flora	9 (90)	1 (50)	2 (100)	NS
Contact lost	1 (10)	1 (50)	0 (0)	
Subjective	2 (10.5)	1 (7.7)	-	
Mothers reporting non-tolerance	2 (100)	0 (0)	-	
Parents believe that study formula did not feed	0 (0)	1 (100)	-	NS

	Control N (%)	SYN1 N (%)	Breastfed N (%)	Control vs. SYN1
Lack of confidence	0 (0)	0 (0)	-	
Introduction complementary feeding	0 (0)	4 (30.8)	0 (0)	
Others	0 (0)	1 (7.7)	0 (0)	
Breastfeeding abandonment	-	-	5 (62.5)	
MONTH 3				
Symptoms	2 (50)	4 (50)	0 (0)	
Vomiting & gastroesophagical reflux	1 (50)	2 (50)	0 (0)	
Loose stools	0 (0)	0 (0)	0 (0)	
Lactose or cow's milk intolerance	1 (50)	2 (50)	0 (0)	NS
Colic	0 (0)	0 (0)	0 (0)	
Lack of weight gain	0 (0)	0 (0)	0 (0)	
Constipation	0 (0)	0 (0)	0 (0)	
Exclusion criteria	1 (25)	2 (25)	6 (54.5)	
Antibiotics/ drugs that may influence flora	1 (100)	2 (100)	5 (83.3)	NS
Contact lost	0 (0)	0 (0)	1 (16.7)	
Subjective	0 (0)	0 (0)	-	
Mothers reporting non-tolerance	0 (0)	0 (0)	-	
Parents believe that study formula did not feed	0 (0)	0 (0)	-	-
Lack of confidence	0 (0)	0 (0)	-	
Others	0 (0)	0 (0)	0 (0)	
Introduction complementary feeding	1 (25)	2 (25)	1 (9.1)	
Breastfeeding abandonment	-	-	4 (36.4)	
MONTH 4				
Symptoms	1 (9.1)	0 (0)	0 (0)	

	Control N (%)	SYN1 N (%)	Breastfed N (%)	Control vs. SYN1
Vomiting & gastroesophagical reflux	0 (0)	0 (0)	0 (0)	
Loose stools	0 (0)	0 (0)	0 (0)	
Lactose or cow's milk intolerance	0 (0)	0 (0)	0 (0)	NS
Colic	1 (100)	0 (0)	0 (0)	
Lack of weight gain	0 (0)	0 (0)	0 (0)	
Constipation	0 (0)	0 (0)	0 (0)	
Exclusion criteria	0 (0)	5 (27.8)	4 (36.4)	
Antibiotics/ drugs that may influence flora	0 (0)	5 (100)	4 (100)	NS
Contact lost	0 (0)	0 (0)	0 (0)	
Subjective	2 (18.2)	1 (5.6)	-	
Mothers reporting non-tolerance	1 (50)	1 (100)	-	
Parents believe that study formula did not feed	0 (0)	0 (0)	-	NS
Lack of confidence	1 (50)	0 (0)	-	
Introduction complementary feeding	8 (72.7)	12 (66.7)	5 (45.5)	-
Others	0 (0)	0 (0)	0 (0)	-
Breastfeeding abandonment	-	-	2 (18.2)	-

Table 16. Summary of drop out reasons.

	Control N (%)	SYN1 N (%)	Breastfed N (%)	Control vs. SYN1
WHOLE STUDY PERIOD				
Symptoms	19 (33.9)	22 (33.8)	2 (2.53)	
Vomiting & gastroesophagical reflux	8 (42.11)	9 (40.90)	0 (0)	
Loose stools	4 (21.05)	1 (4.55)	0 (0)	NS
Lactose or cow's milk	2 (10.53)	8 (36.36)	0 (0)	
Colic	4 (21.05)	1 (4.55)	0 (0)	
Lack of weight gain	1 (5.26)	2 (9.09)	2 (100)	
Constipation	0 (0)	1 (4.55)	0 (0)	
Exclusion criteria	16 (28.6)	15 (23.1)	32 (40.51)	
Antibiotics/ drugs that may	13 (81.3)	12 (80)	20 (62.5)	NS
Contact lost	3 (18.7)	3 (20)	12 (37.5)	
Subjective	11 (19.6)	8 (12.31)	-	
Mothers reporting non-	6 (54.5)	4 (50)		
Parent's believe that study formula did not feed	2 (18.2)	2 (25)		NS
Lack of confidence	3 (27.3)	2 (25)		
Introduction complementary feeding	9 (16.1)	19 (29.2)	6 (7.59)	-
Others	1 (1.8)	1 (1.5)	4 (5.06)	-
Breastfeeding abandonment	-	-	35 (44.30)	

Longitudinal analysis

Withdrawal reasons between formulas were similar over the whole study period. The Cox regressions analyses showed no effect of feeding SYN1 supplemented formula versus control formula to drop out the study.

5.3. CROSS-SECTIONAL AND LONGITUDINAL ANALYSES

5.3.1. Formula intake

Cross-sectional analysis

Average total formula intake and the formula and energy intake per kg body weight were comparable between the two formula groups (Table 17). Therefore, as nutrient composition was the same in the two formulas the energy and nutrient intake were similar in both groups, except for SYN1 and maltodextrin.

Table 17. Description of formula dietary intake by feeding group.

	Control Mean (SD)	**SYN1** Mean (SD)	**Control vs. SYN1**
Month 1	(n=90)	(n=88)	
Formula intake (ml)	775 (139)	786 (161)	0.644
Formula intake (ml/Kg)	184.5 (29.7)	188.3 (34.2)	0.434
Energy intake from formula (kcal/kg)	123.27 (19.82)	122.21 (22.22)	0.736
Month 2	(n=79)	(n=85)	
Formula intake (ml)	857 (129)	859 (155)	0.949
Formula intake (ml/Kg)	165.5 (23.3)	166.6 (24.5)	0.770
Energy intake from formula (kcal/kg)	110.57 (15.58)	108.14 (15.93)	0.324
Month 3	(n=77)	(n=77)	
Formula intake (ml)	921 (188)	919 (176)	0.939
Formula intake (ml/Kg)	153.3 (30.9)	152.9 (24.0)	0.945
Energy intake from formula (kcal/kg)	102.38 (20.68)	99.27 (15.55)	0.293
Month 4	(n=64)	(n=58)	

	Control Mean (SD)	SYN1 Mean (SD)	Control vs. SYN1
Formula intake (ml)	955 (195)	971 (187)	0.635
Formula intake (ml/Kg)	140.8 (26.4)	144.2 (25.8)	0.470
Energy intake from formula (kcal/kg)	94.04 (17.66)	93.60 (16.74)	0.886

Longitudinal analysis

The results of the longitudinal analyses (one-way linear model for repeated measures) showed a significant increase in average formula intake (total amount in ml, energy and energy/Kg body weight) from the first to the 4^{th} month course in both groups ($p<0.001$), which did not differ between feeding groups. The average total formula intake, the energy intake and energy per kg body weight were similar between formula groups.

Introduction of study formula was at median age day 3 (IQR: 2, 5) for infants in control group and 3 (IQR: 2, 7) for infants in SYN1 group (no statistically significant differences between feeding groups were observed).

5.3.2. Anthropometrical evaluation

Cross sectional analysis

Anthropometrical variables were similar between formula groups at all time points (Table 18).

Table 18. Anthropometric characteristics of infants by feeding groups at all timepoints.

	Control Mean (SD)	**SYN1** Mean (SD)	**Breastfeed** Mean (SD)	**Control vs. SYN1**
MONTH 1	(n=102)	(n=102)	(n=87)	
Weight (g)	4184 (433)	4168 (446)	4318 (484)	0.795
Length (cm)	53.8 (1.9)	54.1 (2)	54.4 (1.6)	0.397
Head circumference (cm)	37.3 (1.1)	37.2 (1.3)	37.5 (1.1)	0.714
Waist circumference (cm)	36.4 (2.1)	36.2 (2.1)	37 (1.9)	0.510
Arm circumference (cm)	11.3 (0.8)	11.3 (0.7)	11.5 (0.9)	0.693
Tricipital skinfold (mm)	6.4 (1)	6.2 (0.9)	6.6 (1)	0.072
Subscapular skinfold	6.6 (1)	6.4 (1.1)	6.7 (1.2)	0.151
MONTH 2	(n=83)	(n=89)	(n=79)	
Weight (g)	5165 (523)	5162 (538)	5217(611)	0.972
Length (cm)	57.4 (1.9)	57.6 (2)	57.8 (1.7)	0.661
Head circumference (cm)	39 (1.1)	39 (1.3)	39.2 (1.1)	0.836
Waist circumference (cm)	39 (2.4)	39.1 (2.3)	39 (2.4)	0.817
Arm circumference (cm)	12.3 (0.9)	12.3 (0.8)	12.4 (1)	0.933
Tricipital skinfold (mm)	7.3 (1.2)	7.2 (1)	7.3 (1)	0.806
Subscapular skinfold	7.1 (1.2)	7.1 (1.3)	7.2 (1.1)	0.947
MONTH 3	(n=79)	(n=81)	(n=68)	
Weight (g)	6007 (579)	6003 (636)	5902 (732)	0.963
Length (cm)	60.7 (1.9)	60.8 (2.1)	60.7 (1.8)	0.811

Head circumference (cm)	40.4 (1.2)	40.4 (1.3)	40.5 (1.2)	0.902
Waist circumference (cm)	40.7 (2.3)	40.8 (2.4)	40 (2.4)	0.805
Arm circumference (cm)	13.2 (0.8)	13.1 (0.8)	13.1 (1.1)	0.191
Tricipital skinfold (mm)	8.1 (1.4)	7.9 (1.3)	7.9 (1.3)	0.361
Subscapular skinfold	7.2 (1.1)	7.2 (1.3)	7.3 (1.3)	0.938
MONTH 4	(n=68)	(n=63)	(n=57)	
Weight (g)	6741 (618)	6746 (728)	6491 (797)	0.967
Length (cm)	63.6 (1.8)	63.5 (2.4)	63.2 (2)	0.877
Head circumference (cm)	41.6 (1.2)	41.7 (1.4)	41.6 (1.3)	0.507
Waist circumference (cm)	41.7 (2)	42.2 (2.5)	41 (2.4)	0.166
Arm circumference (cm)	13.8 (0.8)	13.7 (0.82)	13.5 (1)	0.578
Tricipital skinfold (mm)	8.5 (1.5)	8.3 (1.5)	8.5 (1.7)	0.470
Subscapular skinfold	7.4 (1.2)	7.2 (1.3)	7.1 (1.4)	0.276

Longitudinal analysis

Anthropometrical variables increased in all groups throughout the study, and no differences for any of the anthropometrical parameters were seen between formula groups in the whole study period (Table 19).

The anthropometrical data were similar to those reported for the World Health Organization for all feeding groups (149;150).

Table 19. Anthropometric characteristics by feeding group and timepoint for longitudinal analyses.

		Control		SYN1		Breastfed	
		mean (SD)	median (IQR)	mean (SD)	median (IQR)	mean (SD)	median (IQR)
		n: 68		n: 63		n: 57	
Weight (g)	V1	4248 (419)	4280 (3921, 4523)	4245 (410)	4220 (3990,4570)	4299 (484)	4330 (3955,4680)
	V2	5188 (475)	5185 (4876, 5500)	5215 (531)	5190 (4800,5560)	5218 (594)	5150 (4785,5705)
	V3	6021 (550)	6115.0 (5560, 6348)	6039 (661)	6000 (5490,6570)	5908 (725)	5880 (5375,6400)
	V4	6741 (619)	6810 (6231 ,7178)	6746 (728)	6700 (6310,7405)	6491 (797)	6570 (5875,7083)
Length (cm)	V1	54.1 (1.8)	54.4 (52.5,55.3)	54.2 (1.9)	54.5 (52.7,55.2)	54.36 (1.7)	54.5 (53,55.7)
	V2	57.6 (1.7)	57.4 (56.5,58.8)	57.6 (2.0)	58 (56.2,58.7)	57.7 (1.7)	57.3 (56.4,59)
	V3	60.8 (1.8)	60.5 (59.5,62.0)	60.8 (2.2)	60.9 (59,62.5)	60.6 (1.8)	60.5 (59.1,62)
	V4	63.6 (1.8)	63.5 (62.5,64.9)	63.5 (2.4)	63.7 (62,65)	63.2 (2.0)	63.4 (61.5,64.9)
Head cir-cumference (cm)	V1	37.4 (1.1)	37.5 (36.8,38.2)	37.5 (1.2)	37.5 (36.5,38.4)	37.6 (1.2)	37.8 (36.7,38.4)
	V2	39.1 (1.1)	39 (38.4,39.8)	39.2 (1.2)	39 (38.2,40)	39.2 (1.2)	39.4 (38.2,40.2)
	V3	40.4 (1.1)	40.4 (39.5,41.1)	40.5 (1.3)	40.4 (39.6,41.6)	40.6 (1.2)	40.6 (39.6,41.6)
	V4	41.6 (1.2)	41.6 (40.6,42.4)	41.7 (1.4)	41.5 (40.7,42.6)	41.6 (1.3)	41.5 (40.6,42.6)

		Control		SYN1		Breastfed	
		mean (SD)	median (IQR)	mean (SD)	median (IQR)	mean (SD)	median (IQR)
Waist circumference (cm)	V1	36.5 (2)	36.6 (35.3,37.9)	36.5 (2.1)	36.7 (35.2,37.7)	37.1 (1.9)	36.9 (35.5,38.8)
	V2	38.9 (2.1)	39.3 (37.4,40.9)	39.3 (2.2)	39.2 (37.8,40.9)	39.2 (2.4)	39.5 (37.3,40.8)
	V3	40.6 (2.0)	40.7 (39.5,41.6)	41.0 (2.4)	40.5 (39.4,42.8)	40.1 (2.3)	40.5 (38.2,41.8)
	V4	41.7 (2)	42 (40,43.1)	42.2 (2.5)	42 (40.7,44.2)	41 (2.4)	41.1 (39.3,42.6)
Arm circumference (cm)	V1	11.4 (0.8)	11.4 (10.9,11.9)	11.4 (*0.7*)	11.4 (11.0,11.9)	11.4 (0.9)	11.5 (10.8,10.0)
	V2	12.4 (0.8)	12.4 (11.8,13.1)	12.4 (0.7)	12.4 (11.8,13)	12.5 (1.0)	12.4 (11.7,13.5)
	V3	13.3 (0.8)	13.3 (12.7,13.9)	13.1 (0.8)	13.2 (12.4,13.7)	13.2 (1.0)	13.3 (12.5,14.0)
	V4	13.8 (0.8)	13.9 (13.3,14.5)	13.7 (0.8)	13.7 (13.3,14.3)	13.5 (1.0)	13.7 (12.9,14.3)
Tricipital skinfold (mm)	V1	6.4 (0.9)	6.3 (5.9,7.1)	6.2 (0.9)	6.1 (5.7,6.8)	6.6 (1.0)	6.7 (5.9,7.3)
	V2	7.3 (1.2)	7.2 (6.4,8)	7.2 (1.0)	7 (6.5,7.9)	7.4 (1.0)	7.1 (6.7,8.1)
	V3	8.1 (1.3)	7.9 (7.2,9.1)	7.8 (1.5)	7.7 (7,8.3)	8.0 (1.3)	7.9 (7,8.9)
	V4	8.5 (1.5)	8.1 (7.3,9.7)	8.3 (1.5)	8.3 (7.2,9.1)	8.5 (1.7)	8.3 (7.2,9.5)
Subscapular skinfold (mm)	V1	6.6 (1)	6.6 (6,7.1)	6.5 (1.2)	6.2 (5.7,7.2)	6.7 (1.3)	6.8 (5.8,7.3)
	V2	7 (1.1)	7 (6.4,7.6)	7.1 (1.4)	7 (6.3,8)	7.2 (1.2)	7.2 (6.3,8.1)
	V3	7.2 (1.0)	7.2 (6.4,7.9)	7.2 (1.3)	7 (6.3,8.0)	7.3 (1.4)	7 (6.4,8.3)
	V4	7.4 (1.2)	7.2 (6.7,8.1)	7.2 (1.3)	7.1 (6.1,8.2)	7.1 (1.4)	7 (6.3,8.0)

5.3.3. Urinary laboratory analyses

Cross-sectional analysis

At month 1, levels of urinary parameters did not show any significant differences between formula groups, except for potassium, that was significantly lower in the SYN1 group as compared to the control group, but closer to the breastfed group. At month 3, the two formula groups did not differ in any of the urinary parameters analysed. Table 20 shows the results per feeding groups and time point.

Table 20. Description of laboratory urinary parameters by feeding groups.

	Control Median (IQR)	**SYN1** Median (IQR)	**Breastfed** Median (IQR)
MONTH 1	(n=70)	(n=67)	(n=65)
Urinary Cl (mEq/L)	19 (11, 30.3)	17 (10,26)	12 (7,19)
Urinary K (mEq/L)	20.5 (12.7, 29.1)	15.5 (9.9, 23.2)*	12.1 (8.8, 17.3)
Urinary Na (mEq/L)	17 (9.8, 26.3)	12 (7, 23)	5 (3,9)
Urinary Na/K ratio	0.85 (0.6, 1.3)	0.91 (0.5, 1.2)	0.38 (0.2, 0.8)
Urinary Creatinine	8.9 (5.9, 14.4)	8.4 (4.7, 12)	5.9 (4,8.8)
Urinary Osmolarity	150.5 (99.3, 210.3)	115 (80, 192)	90 (54.5, 145)
MONTH 3	(n=59)	(n=60)	(n=49)
Urinary Cl (mEq/L)	22 (9,47)	19.5 (10.3, 31.8)	14 (9,23)
Urinary K (mEq/L)	19.8 (12.3, 33.1)	17.8 (10.7, 27.9)	13.6 (8.7, 22.1)
Urinary Na (mEq/L)	22 (7,50)	17 (7.3, 40.8)	5 (2.5, 10.5)
Urinary Na/K ratio	0.83 (0.43, 1.73)	1.1 (0.6, 1.5)	0.35 (0.16, 0.74)
Urinary Creatinine	11.8 (6.5, 18.9)	9.4 (5.9, 17.5)	9 (5.8, 13.3)
Urinary Osmolarity	159 (6.8, 267.8)	143 (3.8, 253.3)	104 (0, 172)

*p<0.05 vs. Control formula.

The concentrations of these urinary parameters were comparable to the concentration assessed in infants by other groups (151-153).

The Na/K ratio was calculated to evaluate hyperaldosteronism. No significant differences were seen between formulas (neither at 1 month nor at 3 months).

Longitudinal analysis

The two-way linear model for repeated measures confirmed comparable urinary performance with both formulas apart from mean urinary chloride levels ($p<0.05$) and mean urinary sodium levels ($p<0.05$) (Table 21). Both variables were closer to the breastfed group in the SYN1 than control group.

Table 21. Urinary biochemical parameters by feeding group and timepoint for longitudinal analyses.

		Control (C)			SYN1 (S)			C vs. S	Breastfed	
		mean (SD)	Median (IQR)	P value	mean (SD)	Median (IQR)	P value	P value	mean (SD)	Median (IQR)
			n: 57			n: 53				n: 50
Urinary Cl (mEq/L)	V1	24.4 (17.6)	20 (11,32)	0.058	18.6 (13.9)	16 (7,25.5)	0.122	**0.013**	14.4 (10.1)	12 (8.8,18)
	V3	31.1 (29.8)	22 (8.8,44.8)		25.2 (21.2)	19 (10.5,31.5)			16.6 (11.1)	14 (9,20)
Urinary K (mEq/L)	V1	23.2 (21)	21 (12.9,32.5)	0.453	18.7 (12.7)	15.4 (9.7,23.9)	0.412	0.059	14.0 (6.9)	12.3 (9.5,17.3)
	V3	27.6 (25.9)	19.8 (12.3,32.8)		23.9 (19.8)	17.3 (10.8,27.4)			16.5 (10.1)	13.6 (8.5,22.2)
Urinary Na (mEq/L)	V1	34.3 (66.2)	20 (11,28.5)	**0.031**	20.5 (33.5)	11 (6,21.5)	**0.005**	**0.038**	10.7 (20.6)	4.5 (3,8.3)
	V3	52.2 (118.2)	21 (6.8,50.8)		27.1 (26.6)	17 (6.5,40.5)			7.3 (7.6)	4 (2,10)
Urinary Na/K ratio	V1	1.6 (3)	0.9 (0.6,1.3)	0.289	1.0 (1.1)	0.8 (0.5,1.2)	**0.020**	0.169	1 (2.6)	0.4 (0.2,0.7)
	V3	2.9 (10.5)	0.8 (0.4,1.7)		1.3 (1.1)	1.1 (0.5,1.5)			0.6 (0.7)	0.3 (0.1,0.7)

		Control (C)			SYN1 (S)			C vs. S	Breastfed	
		mean (SD)	Median (IQR)	P value	mean (SD)	Median (IQR)	P value	P value	mean (SD)	Median (IQR)
Urinary Creatinine (mEq/dL)	V1	11.7 (7.4)	9.8 (5.9,14.9)	0.083	9.1 (6.2)	8.4 (4.3,11)	0.129	0.183	7.2 (5)	6.2 (4,8.8)
	V3	30.8 (105.4)	10.9 (6.3,18.8)		14.1 (13.1)	9.6 (5.9,16.6)			11.6 (9)	9 (5.8,14.1)
Urinary Osmolarity (mosm/l)	V1	202.0 (170.2)	155 (107,211.5)	0.251	181.3 (201.9)	119 (80,195.5)	0.433	0.071	143.1 (152.3)	96 (59.8,144)
	V3	285.4 (422)	159 (96.5,276.5)		195.7 (164.9)	144 (74.5,249.5)			135.4 (120.5)	96 (50,171)

5.3.4. Serum laboratory analyses

Blood serum was analysed at the age of 3 months. Most of the assessed safety serum parameters, minerals (Na, K, Cl, Ca-ion), pH, and proteins (total protein, albumin, pre-albumin), were similar in both study formula groups, except urea (Table 22). Serum urea was slightly higher in the control than in the SYN1 group, but in the SYN1 group closer to the breastfed group. Serum base excess and cholesterol were also similar in both formula groups. All these parameters were within the normal range for infants/children.

Table 22. Description of laboratory serum parameters by feeding groups.

	Control Mean (SD)	**SYN1** Mean (SD)	**Breastfed** Mean (SD)	**Control vs. SYN1**
	(n=29)	(n=37)	(n=22)	
Serum Cholesterol	130.2 (15.9)	132.7	157.8 (27.2)	0.796
Serum Urea (mg/dL)	22.62 (3.27)	19.64	13.8 (3.6)	**0.003**
Serum Albumin (g/dL)	4.25 (0.38)	4.23	4.2 (0.24)	0.898
Serum Proteins (g/dL)	6.0 (0.36)	6.0 (0.42)	5.9 (0.37)	0.639
Serum Na (mEq/L)	138.6 (1.5)	138.5	137.8 (2.1)	0.790
Serum K (mEq/L)	5.5 (0.5)	5.4 (0.6)	5.2 (0.4)	0.748
Serum Cl (mEq/L)	105.4 (1.7)	106.1	106.4 (2.1)	0.095
Serum Prealbumin	20.11 (6.89)	21.02	16.3 (5.1)	0.542
Serum pH	7.34 (0.04)	7.33	7.36 (0.06)	0.809
Serum base excess	-1.4 (1.8)	-1.6 (2.1)	-0.5 (2.1)	0.485
Serum Ca_ion (mmol/L)	1.3 (0.1)	1.3 (0.1)	1.3 (0.1)	0.423
IgG (mg/dL)	374.3 (114.7)	366.5	358.6 (77.6)	0.793

	Control Mean (SD)	SYN1 Mean (SD)	Breastfed Mean (SD)	Control vs. SYN1
IgM (mg/dL)*	41.93 (1.60)	52.03	52.08 (1.59)	0.130
IgE (UI/mL)	11.2 (9.6)	10.9	7.3 (3.4)	0.946
IgG Diphteria (UI/mL)	0.2 (0.1)	0.3 (0.6)	0.3 (0.4)	0.609
IgG Tetanus (UI/mL)*	0.25 (2.50)	0.34	0.38 (3.30)	0.387

The number of infants below/above the detection limit for C-reactive protein was similar in both formula groups (Table 23). Detection of IgG to Diphteria and IgG to Pertusis didn't differ between formula groups, while, IgE response was significantly higher in control group (<0.01).

Table 23. Frequency of laboratory serum parameters below and above the detection limit by feeding group.

	Control N (%)	SYN1 N (%)	Breastfed N (%)	Control vs. SYN1
Serum C-reactive protein (over detection limit)				
Yes	1 (3.4)	0 (0)	4 (18.2)	0.255
No	28 (96.6)	37 (100)	18 (81.8)	
IgG Diphteria cat (over detection limit)				
Yes	4 (16.7)	10 (30.3)	10 (47.6)	0.238
No	20 (83.3)	23 (69.7)	11 (52.4)	
IgE response (over detection limit)				
Yes	14 (50)	6 (16.7)	2 (9.5)	**0.004**
No	14 (50)	30 (83.3)	19 (90.5)	
IgG Pertusis (over detection limit)				
Yes	6 (22.2)	8 (22.9)	7 (33.3)	0.953
No	21 (77.8)	27 (77.1)	14 (66.7)	

5.3.5. Study formula acceptance

Mother's perception of infant's formula acceptance was similar in both formula groups at all time points (Table 24).

Table 24. Mother's perception of infants' acceptance of study formula.

	Control N (%)	**SYN1** N (%)	**Control vs. SYN1**
1 month - Infants acceptance	n= 64	n= 64	
Very good	50 (78.1)	48 (75)	
Good	14 (21.9)	15 (23.4)	
Moderate	0 (0)	1 (1.6)	0.584
Poor	0 (0)	0 (0)	
Very poor	0 (0)	0 (0)	
2 month - Infants acceptance	n= 62	n= 65	
Very good	41 (66.1)	47 (72.3)	
Good	20 (32.3)	18 (27.7)	
Moderate	1 (1.6)	0 (0)	0.486
Poor	0 (0)	0 (0)	
Very poor	0 (0)	0 (0)	
3 month - Infants acceptance	n= 60	n= 61	
Very good	51 (85)	53 (86. 9)	
Good	9 (15)	7 (11.5)	
Moderate	0 (0)	1 (1.6)	0.527
Poor	0 (0)	0 (0)	
Very poor	0 (0)	0 (0)	
4 month - Infants acceptance	n= 49	n= 46	
Very good	46 (93.9)	39 (84.8)	
Good	3 (6.1)	7 (15.2)	
Moderate	0 (0)	0 (0)	0.149
Poor	0 (0)	0 (0)	
Very poor	0 (0)	0 (0)	

5.3.6. Bowel function and digestive symptoms

Results of the 2 day diary

Cross-sectional analysis

At all timepoints, the SYN1-fed infants had significantly higher stools numbers than infants fed with the standard formula; and the numbers of the firsts were closer to that of the breastfed group at months 1 and 2. At all timepoints, the consistency of the stools was softer in the SYN1 than in the control and closer to the breastfed group. (Table 25) (Figures 11 and 12).

Table 25. Digestive symptoms by feeding group and timepoint.

	Control Median (IQR)	**SYN1** Median (IQR)	**Breastfed** Median (IQR)	**Control vs. SYN1**
MONTH 1	(n=97)	(n=102)	(n=84)	
Depositions n	2.5 (2.0, 4.0)	4 (2.5, 5.1)	5 (2.5, 7.0)	**<0.001**
Regurgitation n	2 (0, 3.5)	1 (0, 2.6)	2.5 (0.5, 4.5)	0.125
Vomits n	0 (0,0)	0 (0, 0)	0 (0, 0)	0.745
Faecal consistency	6 (4.7, 6.0)	6 (6, 6)	6.3 (6, 7)	**<0.001**
MONTH 2	(n=81)	(n=87)	(n=77)	
Depositions n	2 (1.5, 3.0)	2.5 (2, 3.5)	3 (1.5, 5)	**0.009**
Regurgitation n	0.5 (0, 3.3)	0.5 (0, 2.5)	1.5 (0, 4)	0.493
Vomits n	0 (0, 0)	0 (0, 0)	0 (0, 0)	0.407
Faecal consistency	6 (4.3, 6)	6 (6, 6)	6.8 (6, 7)	**0.001**
MONTH 3	(n=78)	(n=81)	(n=67)	
Depositions n	2 (1.5, 2.5)	2.5 (1.5, 3)	2 (1, 4)	**0.005**
Regurgitation n	0.5 (0, 2.5)	0.5 (0, 2.3)	1.5 (0, 2.5)	0.881
Vomits n	0 (0,0)	0 (0,0)	0 (0,0)	0.229
Faecal consistency	6 (4, 6)	6 (6, 6)	6.5 (6, 7)	**<0.001**
MONTH 4	(n=66)	(n=62)	(n=54)	

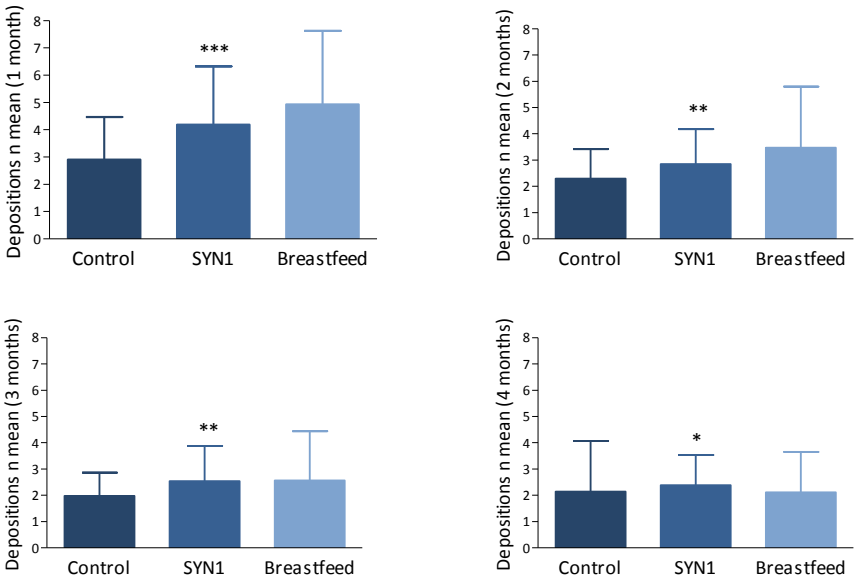
	Control Median (IQR)	SYN1 Median (IQR)	Breastfed Median (IQR)	Control vs. SYN1
Depositions n	2 (1.5, 2.5)	2.5 (1.5, 3)	1.5 (1, 3)	**0.046**
Regurgitation n	0.5 (0, 2)	0 (0, 1.3)	0.8 (0, 2.8)	0.272
Vomits n	0 (0, 0)	0 (0, 0)	0 (0, 0)	0.072
Faecal consistency	6 (4,6)	6 (6,6)	6.5 (6,7)	**<0.001**

*This variable was calculated in order to obtain the mean consistency of the total stools reported in the 2-day diary (using the Bristol Stools Form Scale) (143).
The formula was:

$$Fecal\ consistency\ score = \frac{(a \times 7) + (b \times 6) + \ldots\ldots\ldots + (f \times 2) + (g \times 1)}{a + b + c + d + e + f + g}$$

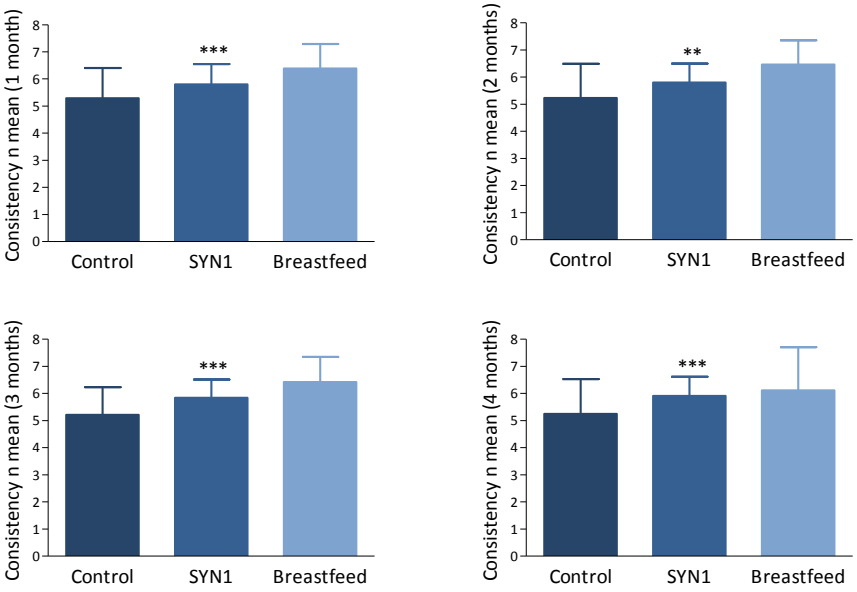
a: number of depositions type 7, b: number of depositions type 6, c: number of depositions type 5, d: number of depositions type 4, e: number of depositions type 3, f: number of depositions type 2, g: number of depositions type 1. The higher is the number of Bristol Scale (from 1 to 7), softer is the stool consistency.

Figure 11. Number of mean depositions per day in both formula groups and the breastfed infants, during the 4 first months of life.



***p<0.001, **p<0.01 and *p<0.05 vs. control group.

Figure 12. Faecal consistency score in both formula groups and the breastfed reference infants, during the 4 first months of life.



***p<0.001 vs. control group, **p=0.01 vs. control group.

Longitudinal analysis

Stools parameters differed significantly between formula groups for number of depositions (inter-subject, p<0.01) and consistency (inter-subject, p<0.001) (Table 26). In the SYN1 group the stools numbers were significantly higher and stools significantly softer compared to the control group. Regurgitation and vomiting occurred similarly in both formula groups.

Table 26. Description of digestive symptoms (data from the parents' diary) for longitudinal analyses.

		Control (C)			SYN1 (S)			C vs. S	Breastfed	
		Mean (SD)	Median (IQR)	P value	Mean (SD)	Median (IQR)	P value	P value	Mean (SD)	Median (IQR)
Depositions n mean	V1	2.9 (1.6)	2.3 (2,4)	**<0.001**	3.9 (1.9)	3.5 (2.5,4.5)	**<0.001**	**0.003**	4.9 (2.5)	5 (2.6,7)
	V2	2.4 (1.1)	2.3 (1.5,3)		2.9 (1.4)	2.5 (2,3.5)			3.6 (2.1)	3.5 (2,5)
	V3	2 (0.9)	2 (1.5,2.5)		2.6 (1.4)	2.5 (1.5,3)			2.6 (1.9)	2 (1,4)
	V4	2.1 (1.9)	2 (1.5,2.5)		2.4 (1.2)	2.5 (1.5,3)			2.1 (1.5)	1.5 (1,3)
Regurgita-tion n mean	V1	2.8 (3.3)	2 (0,4)	**0.050**	1.9 (3.2)	0.5 (0,2)	**0.020**	0.099	4 (4.2)	3 (0.5,5.8)
	V2	2.1 (3.5)	0.8 (0,3.5)		1.5 (2.6)	0 (0,2)			2.5 (3.3)	1.5 (0,4)
	V3	2.4 (4.3)	0.5 (0,2.4)		1.6 (2.5)	0.5 (0,2)			2.3 (3.4)	1.5 (0,2.9)
	V4	2.2 (4.2)	0.5 (0,2)		1.4 (2.5)	0 (0,1.3)			2 (2.9)	0.8 (0,2.8)
Vomits n mean	V1	0.1 (0.3)	0 (0,0)	**0.001**	0.1 (0.3)	0 (0,0)	0.126	0.676	0.1 (0.5)	0 (0,0)
	V2	0.1 (0.4)	0 (0,0)		0.1 (0.4)	0 (0,0)			0.1 (0.5)	0 (0,0)
	V3	0.0 (0.1)	0 (0,0)		0.1 (0.3)	0 (0,0)			0.1 (0.2)	0 (0,0)
	V4	0 (0)	0 (0,0)		0 (0.2)	0 (0,0)			0.0 (0.2)	0 (0,0)
Faecal consistency score	V1	5.3 (1.1)	6 (4.8,6)	0.713	5.9 (0.6)	6 (6,6)	**0.040**	**<0.001**	6.4 (0.6)	6.2 (6,7)
	V2	5.3 (1.1)	6 (4.3,6)		5.8 (0.7)	6 (6,6)			6.5 (0.5)	6.7 (6,7)
	V3	5.2 (1.0)	6 (4,6)		5.9 (0.6)	6 (6,6)			6.4 (1)	6.4 (6,7)
	V4	5.3 (1.3)	6 (4,6)		5.9 (0.7)	6 (6,6)			6.1 (1.6)	6.5 (6,7)

Results of the mother's interview at visits (recall 4 weeks) on frequency of depositions and digestive symptoms

Cross-sectional analysis on frequency of depositions recalled by mothers

More mothers in the SYN1 group reported significantly higher stools frequency stools compared to mothers in the control group at each time point. SYN1 formula fed children showed a more similar pattern to breastfed infants (Table 27).

Table 27. Frequency of depositions recalled by mothers by feeding group by timepoint.

	Control Median (IQR)	**SYN1** Median (IQR)	**Breastfed** Median (IQR)	**Control vs. SYN1**
MONTH 1	(n=101)	(n=102)	(n=86)	
Depositions/week	21 (14, 28)	28 (14, 35)	35 (21, 42)	**0.000**
MONTH 2	(n=82)	(n=88)	(n=77)	
Depositions/week	14 (7, 21)	21 (14, 28)	21 (14, 35)	**0.004**
MONTH 3	(n=79)	(n=81)	(n=67)	
Depositions/week	14 (7, 14)	14 (14, 21)	14 (7, 28)	**0.001**
MONTH 4	(n=68)	(n=63)	(n=57)	
Depositions/week	14 (7, 14)	14 (7, 21)	14 (7, 21)	**0.031**

Longitudinal analysis on frequency of depositions recalled by mothers

The longitudinal analysis showed that the number of depositions (recalled by the mothers at study visits) diminished with the infant's age in both formula groups ($p<0.001$, both groups) (Table 28). The 2-way ANOVA for repeated measures showed significant inter-group differences ($p<0.001$).

Table 28. Frequency of depositions recalled by mothers by feeding group and timepoint for longitudinal analyses (children with available data at all visits).

Type of feeding		mean (SD)	Median (IQR)	P value
CONTROL		n: 68		
Number of depositions/week	V1	20.2 (11.3)	17.5 (14,28)	**<0.001**
	V2	16.6 (11.3)	14 (14,21)	
	V3	13.8 (6.7)	14 (14,21)	
	V4	12.9 (5.4)	14 (7,14)	
SYN1		n: 63		
Number of depositions/week	V1	26.1 (12.6)	28 (14,35)	**<0.001**
	V2	20 (9.3)	21 (14,28)	
	V3	17.7 (9.4)	14 (14,21)	
	V4	15.8 (7.6)	14 (7,21)	
BF		n: 57		
Number of depositions/week	V1	34 (17.4)	35 (21,47.3)	**<0.001**
	V2	25.2 (14.8)	28 (14,38.5)	
	V3	19.6 (13.7)	14 (7,28)	
	V4	14.8 (11.4)	14 (7,21)	

*SYN1 supplemented infants had an overall higher frequency than not-supplemented (control) infants (p<0.001).

Cross sectional analysis on frequency of digestive symptoms

Mother's perception of infant's frequency in digestive discomfort, vomits or regurgitation (Table 29) was similar in both formula groups at every timepoint.

Table 29. Mother's recall of digestive symptoms the previous 4 weeks.

		Control n (%)	SYN1 n (%)	BF n (%)	Control vs. SYN1
MONTH 1					
Frequent digestive discomfort	Yes	39 (38.2)	27 (26.5)	34 (40.5)	0.073
	No	63 (61.8)	75 (73.5)	50 (59.5)	
Frequent vomits	Yes	1 (0.99)	5 (4.90)	4 (4.7)	0.100
	No	100 (99)	97 (95.1)	82 (95.3)	
Frequent regurgitations	Yes	10 (9.9)	13 (12.8)	16 (18.6)	0.523
	No	91 (90.1)	89 (87.3)	70 (81.4)	
MONTH 2					
Frequent digestive discomfort	Yes	19 (23.5)	15 (17)	13 (16.5)	0.299
	No	62 (76.5)	73 (83)	66 (83.5)	
Frequent vomits	Yes	1 (1.2)	5 (5.6)	1 (1.3)	0.122
	No	80 (98.7)	84 (94.4)	77 (98.7)	
Frequent regurgitations	Yes	8 (9.9)	11 (12.5)	9 (11.7)	0.590
	No	73 (90.1)	77 (87.5)	68 (88.3)	
MONTH 3					
Frequent digestive discomfort	Yes	2 (2.60)	5 (6.3)	5 (7.4)	0.268
	No	75 (97.40)	75 (93.8)	62 (9.7)	
Frequent vomits	Yes	0 (0)	2 (2.5)	1 (1.5)	0.157
	No	79 (100)	78 (97.5)	66 (98.5)	
Frequent regurgitations	Yes	11 (14.1)	9 (11.4)	8 (11.9)	0.611
	No	67 (85.9)	70 (88.6)	59 (88.1)	
MONTH 4					
Frequent digestive discomfort	Yes	1 (1.5)	2 (3.2)	2 (3.5)	0.514
	No	66 (98.5)	60 (96.8)	55 (96.5)	
Frequent vomits	Yes	0 (0)	1 (1.6)	1 (1.8)	0.293
	No	68 (100)	61 (98.4)	56 (98.2)	
Frequent regurgitations	Yes	6 (9)	5 (8.1)	6 (10.5)	0.856
	No	61 (91)	57 (91.9)	51 (89.5)	

5.3.7. Infant's illness

The number of infectious episodes did not differ between formula groups, neither infections nor non-infection episodes at all time points (Table 30).

Table 30. Description of illness episodes by feeding groups.

	Control Mean (SD)	**SYN1** Mean (SD)	**BF** Mean (SD)	**Control vs. SYN1**
MONTH 1	(n=99)	(n=102)	(n=86)	
infection episode (n)	0.09 (0.28)	0.16 (0.39)	0.12 (0.32)	0.214
non-infection episode (n)	0.00 (0.00)	0.00 (0.00)	0.01 (0.11)	1.000
MONTH 2	(n=83)	(n=89)	(n=79)	
infection episode (n)	0.14 (0.39)	0.19 (0.42)	0.11 (0.32)	0.403
non-infection episode (n)	0.00 (0.00)	0.00 (0.00)	0.03 (0.16)	1.000
MONTH 3	(n=78)	(n=81)	(n=68)	
infection episode (n)	0.17 (0.38)	0.21 (0.44)	0.07 (0.26)	0.592
non-infection episode (n)	0.00 (0.00)	0.01 (0.11)	0.00 (0.00)	0.326
MONTH 4	(n=67)	(n=63)	(n=56)	
infection episode (n)	0.13 (0.34)	0.21 (0.51)	0.11 (0.31)	0.621
non-infection episode (n)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	1.000

5.3.8. Atopic dermatitis

There were no significant differences in the presence of atopic dermatitis between formula groups at every single timepoint (Table 31).

Table 31. Presence of atopic dermatitis (n cases).

	Control N (%)	SYN1 N (%)	BF N (%)	Control vs. SYN1
MONTH 1				
Presence of Atopic Dermatitis	1 (1)	1 (1)	7 (8.3)	1.000
MONTH 2				
Presence of Atopic Dermatitis	4 (5.1)	4 (4.8)	6 (7.7)	0.929
MONTH 3				
Presence of Atopic Dermatitis	3 (3.9)	3 (3.8)	3 (4.6)	0.974
MONTH 4				
Presence of Atopic Dermatitis	4 (6.3)	3 (4.8)	3 (5.3)	0.713

5.3.9. Child's behaviour

The questionnaire on Child's behaviour was part of the "Parent's diary" and was filled in by the parents at home.

Cross-sectional analysis

Both formulas showed similar results regarding crying episodes and total crying time at all time points apart from a slightly higher crying time in the SYN1 group compared to the control group at month 4 that might be due to a low level with the standard formula (Table 32).

Longitudinal analysis

One-way linear models for repeated measures did not show any significant difference on infant's crying episodes and total crying time between feeding groups.

Table 32. Mother's report of episodes and time crying per day.

	Control (C) Median (IQR)	**SYN1 (S)** Median (IQR)	**BF** Median (IQR)	**C vs. S**
MONTH 1	(n=90)	(n=98)	(n=82)	
Crying episodes	6 (3.75,8)	5 (4,8)	6 (3,8)	0.585
Crying time	41 (17.5,80.75)	35.5 (20,80)	30 (15,60)	0.913
MONTH 2	(n=77)	(n=85)	(n=76)	
Crying episodes	4 (2,6)	4 (2,6)	5 (2,6.75)	0.842
Crying time	26 (8.5,62.5)	25 (10,42.5)	25 (10.25,50)	0.844
MONTH 3	(n=75)	(n=81)	(n=67)	
Crying episodes	4 (2,6)	3 (2,6)	3 (2,5)	0.773
Crying time	19 (6,40)	20 (6,35)	20.5 (10,38.75)	0.681
MONTH 4	(n=64)	(n=61)	(n=55)	
Crying episodes	3 (1, 5)	4 (2, 6)	4 (2, 6)	0.154
Crying time	15 (3, 25.7)	20 (10, 39.5)	18 (10, 32)	**0.046**

5.3.10. Analyses of microbiota in infants' faecal samples

At baseline and at month 3, 169 faecal samples from 169 infants were collected. In 8 samples at baseline (6 BF, 1 control, 1 SYN1) and in 9 samples at month 3 (8 BF, 1 control) no DNA extraction was possible because not enough stools material was available. So, DNA extraction and qPCR analysis was performed from 161 samples at baseline and from 160 samples at month 3. For the statistical analysis 9 infants at month 3 (2 BF, 5 control, 2 SYN1) were excluded for antibiotic or laxative treatment during the study.

Accordingly, at baseline 53 infants in the breastfed, 55 in the control and 53 in the SYN1 group and at month three, 49 infants in the breastfed, 50 in the control and 52 in the SYN1 group were included in the cross-sectional data analysis. A complete data set (baseline and month 3) for the longitudinal analysis was available for 44 infants in the breastfed, 49 in the control and 51 in the SYN1 group.

Delivery type is an early factor which might influence colonisation of the infants gut. Thus, the delivery type of infants whose faecal samples were analysed (those who completed the full protocol) is checked (Table 33). In this subsample of infants', the proportion of caesarean sections and vaginal delivery was similar in both formula groups and the breastfed group. Similarly, the delivery types of the subsample from who faecal samples were analysed were similar to the whole study sample.

Table 33. Frequency of caesarean section and vaginal delivery in feeding groups initially recruited and in those whose faecal samples were analysed.

	Control N (%)	SYN1 N (%)	BF N (%)	Control vs. SYN1	BF vs. SYN1	BF vs. Control
AT BASELINE						
Vaginal	37	43 (81.8)	41 (77.4)	0.100	0.632	0.242
Caesarean	18	10 (18.9)	12 (22.6)			
MONTH 3						
Vaginal	34 (68)	42 (80.8)	38 (77.6)	0.139	0.690	0.286
Caesarean	16 (32)	10 (19.2)	11 (22.4)			

The number of analysed faecal bacteria was in several samples below the detection limit, which were 5 log cells/g faeces (Table 34). More than 50% of the samples showed results below the detection limit for C. coccoides and C. leptum, so, these bacteria were omitted (Table 35, 36, 37 and 38).

Table 34. Number of faecal samples having bacteria counts below the detection limit.

	Feeding Group	AT BASELINE n (of total samples)	MONTH 3 n (of total samples)
Bacteroides	Control	12 (55)	8 (50)
	SYN1	21 (53)	15 (52)
	Breastfed	14 (53)	14 (49)
Bifidobacteria	Control	14 (55)	3 (50)
	SYN1	13 (53)	2 (52)
	Breastfed	13 (53)	1 (49)
C. coccoides	Control	39 (55)	28 (50)

	Feeding Group	AT BASELINE n (of total samples)	MONTH 3 n (of total samples)
	SYN1	42 (53)	23 (52)
	Breastfed	48 (53)	38 (49)
C. leptum	Control	51 (55)	28 (50)
	SYN1	50 (53)	31 (52)
	Breastfed	51 (53)	37 (49)
Enterobacteriaceae	Control	27 (55)	2 (50)
	SYN1	26 (53)	4 (52)
	Breastfed	26 (53)	15 (49)
total counts	Control	0 (55)	0 (50)
	SYN1	0 (53)	0 (52)
	Breastfed	0 (53)	0 (49)

Cross-sectional analysis

At baseline all bacteria cell counts were similar in both formula groups, apart from slightly lower total bacteria counts in the SYN1 group (Table 35). At month 3, there was a strong trend for higher Bifidobacteria cell counts in the SYN1 group compared to the control group (p=0.061) and the Bifidobacteria counts were more similar to the breast fed group. The numbers of Bacteroides and Enterobacteriaceae were significantly lower in the SYN1 group than in the control group and more similar to the breastfed group. Total bacteria counts were similar between both formula groups at month 3 (Table 35).

At baseline the proportion and of each of the analyzed bacteria groups of the total bacteria were similar in both formula groups (Table 36).

Table 35. Description of microbiota in faecal samples of formula fed infants. Cross sectional comparison of infants SYN1-supplemented vs. control at baseline and after 3 months of treatment.

log cfu/g feces		Control		SYN1		Breastfed		Control
		mean (SD)	median (IQR)	mean (SD)	Median (IQR)	mean (SD)	Median (IQR)	vs. SYN1
n	V0	55		53		53		
	V3	50		52		49		
Bacteroides	V0	7.36 (2.08)	6.88 (5.22, 9.31)	6.66 (1.96)	6.01 (5.0, 7.8)	7.66 (2.53)	7.1 (5,0, 10.2)	0.060
	V3	8.41 (2.27)	9.15 (5.7, 10.25)	7.32 (2.2)	7.45 (5.0, 9.1)	7.77 (2.53)	7.68 (5.0, 10.32)	**0.012**
Bifidobacteria	V0	6.95 (1.85)	6.29 (5.0, 8.86)	7.09 (1.94)	6.3 (5.04, 9.28)	6.63 (1.72)	6.0 (5.02, 7.59)	0.569
	V3	8.33 (1.9)	8.64 (6.92, 10.02)	9.08 (1.6)	9.59 (8.55, 10.17)	9.45 (1.40)	9.62 (9.16, 10.24)	0.061
Entero-bacteriaceae	V0	7.2 (2.45)	5.11 (5.0, 9.99)	6.74 (2.25)	5.1 (5.0, 9.47)	6.72 (2.1)	5.38 (5.0, 9.38)	0.444
	V3	8.95 (1.32)	9.28 (8.5, 9.88)	8.44 (1.37)	8.71 (7.88, 9.33)	7.64 (2.0)	8.51 (5.0, 9.35)	**0.014**
total counts	V0	10.44 (0.87)	10.6 (10.12, 11.03)	10.11 (0.91)	10.26 (9.57, 10.72)	10.13 (1.38)	10.42 (9.07, 11.08)	**0.030**
	V3	10.88 (1.32)	11.13 (10.37, 11.46)	10.72 (0.88)	10.87 (10.44, 11.32)	10.56 (0.83)	10.67 (9.79, 11.33)	0.272

Cfu: colony-forming unit.

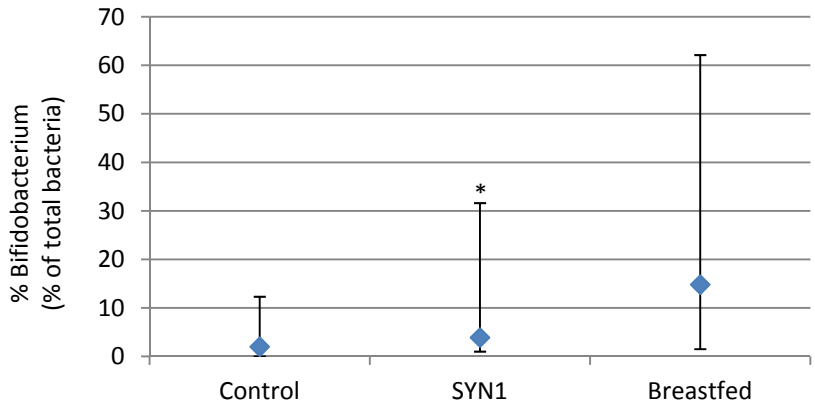
Table 36. Percentage of total bacteria (cross-sectional data). Statistical comparison of SYN1 vs. control group at baseline (V0) and month 3 (V3).

log cfu/g feces		Control		SYN1		Breastfed		Control
		mean (SD)	median (IQR)	mean (SD)	Median (IQR)	mean (SD)	Median (IQR)	vs. SYN1
n	V0	55		53		53		
	V3	50		52		49		
Bacteroides	V0	16.8 (39.2)	0.0 (0.0, 5.2)	5.9 (18.8)	0.0 (0.0, 1.1)	28.8 (56.1)	0.3 (0.0, 33.4)	0.309
	V3	16.2 (34.0)	2.3 (0.0, 13.3)	12.5 (36.2)	0.1 (0.0, 1.0)	22.4 (45.2)	0.8 (0.0, 28.4)	**0.011**
Bifidobacteria	V0	4.5 (11.5)	0.0 (0.0, 2.0)	10.2 (21.4)	0.1 (0.0, 5.5)	8.0 (28.0)	0.0 (0.0, 1.3)	0.430
	V3	10.3 (22.0)	2.0 (0.0, 12.3)	31.0 (60.8)	3.9 (1.0, 31.6)	46.3 (74.4)	14.8 (1.5, 62.1)	**0.022**
Entero-bacteriaceae	V0	12.3 (19.5)	0.0 (0.0, 19.5)	9.0 (16.8)	0.0 (0.0, 10.1)	8.5 (17.5)	0.0 (0.0, 8.3)	0.316
	V3	6.9 (10.6)	2.1 (0.4, 8.5)	3.3 (6.3)	0.7 (0.2, 3.0)	5.5 (12.7)	0.5 (0.0, 4.5)	0.058
total counts	V0	33.9 (41.6)	20.5 (0.1, 51.7)	25.2 (33.0)	14.9 (0.0, 37.1)	48.2 (76.8)	16.7 (0.1, 59.4)	0.212
	V3	36.2 (54.5)	18.4 (5.1, 44.3)	50.6 (69.0)	14.7 (3.2, 69.0)	14.8 (17.4)	10.6 (2.1, 20.8)	0.708

Cfu: colony-forming unit.

At month 3, as previously mentioned, there was a trend toward higher Bifidobacterium cell counts in SYN1 group. When we analysed the proportion of Bifidobacteria (%) in the total bacteria, these differences became significant ($p<0.05$) (Figure 13). The Bifidobacterium counts in the SYN1 group were significantly higher than in the control group and more similar to the breastfed group (Figure 13). The proportion of the Bacteroides was in the SYN1 group lower than in the control group (Table 38), which is in accordance with the lower mean cell counts of Bacteroides in the SYN1 group compared with the control group.

Figure 13. Percentages of Bifidobacterium in the stool samples of the feeding groups at infant's age 3 months.



*p<0.005 vs. control group

Longitudinal analysis

All bacteria cell counts increased from baseline to month 3. We observed a trend in the SYN1 groups (table 37). The proportion of Bifidobacteria increased significantly from baseline to month 3, in both formula groups. The proportion of all other bacteria did not change over time (table 38).

Table 37. Description of microbiota in faecal samples of formula fed infants. Longitudinal data set for log numbers, statistical comparison of baseline (V0) vs. month 3 (V3) in each group.

log cfu/g feces		Control			SYN1			Breastfed		Control vs. SYN1
		mean (SD)	median (IQR)	P value	mean (SD)	Median (IQR)	P value	mean (SD)	Median (IQR)	
n		49			51			44		
Bacteroides	V0	7.33 (2.09)	6.8 (5.29, 9.41)	**0.011**	6.70 (1.98)	6.01 (5.0, 7.87)	0.057	7.68 (2.58)	7.27 (5.0, 10.17)	0.438
	V3	8.36 (2.26)	9.1 (5.64, 10.20)		7.37 (2.19)	7.51 (5.0, 9.11)		7.79 (2.52)	7.8 (5.0, 10.35)	
Bifidobacteria	V0	6.86 (1.82)	5.91 (5.03, 8.75)	**0.000**	7.13 (1.96)	6.3 (5.08, 9.32)	**0.000**	6.59 (1.73)	5.94 (5.0, 7.69)	0.334
	V3	8.35 (1.91)	8.65 (6.68, 10.02)		9.05 (1.6)	9.58 (8.54, 10.16)		9.37 (1.44)	9.66 (8.86, 10.19)	
Entero-bacteriaceae	V0	7.36 (2.46)	5.44 (5.0, 9.99)	**0.001**	6.73 (2.25)	5.1 (5.0, 9.5)	**0.000**	6.66 (2.07)	5.19 (5.0, 9.4)	0.843
	V3	8.96 (1.33)	9.28 (8.5, 9.88)		8.42 (1.38)	8.69 (7.88, 9.33)		7.63 (1.98)	8.48 (5.0, 9.27)	
Total counts	V0	10.48 (0.9)	10.64 (10.13, 11.08)	**0.024**	10.12 (0.93)	10.3 (9.47, 10.74)	**0.001**	10.19 (1.37)	10.42 (9.32, 11.09)	0.408
	V3	10.86 (0.88)	11.12 (10.37, 11.45)		10.71 (0.88)	10.86 (10.44,		10.54 (0.86)	10.7 (9.73, 11.34)	

Cfu: colony-forming unit.

Table 38. Percentage of total bacteria (longitudinal data). Statistical comparison of SYN1 vs. control group at baseline (V0) and month 3 (V3).

log cfu/g feces		Control			SYN1			Breastfed		Control vs. SYN1
		mean (SD)	median (IQR)	P value	mean (SD)	Median (IQR)	P value	mean (SD)	Median (IQR)	
N		49			51			44		
Bacteroides	V0	17.5 (40.9)	0.0 (0.0, 5.2)	0.590	6.1 (19.1)	0.0 (0.0, 1.5)	0.798	32.7 (60.6)	0.2 (0.0, 47.9)	0.125
	V3	16.3 (34.4)	1.8 (0.0, 13.4)		12.7 (36.5)	0.1 (0.0, 1.0)		21.0 (43.7)	1.3 (0.0, 22.0)	
Bifidobacteria	V0	3.3 (8.4)	0.0 (0.0, 1.9)	**0.009**	10.6 (21.7)	0.1 (0.0, 5.6)	**0.005**	9.0 (30.4)	0.0 (0.0, 1.5)	**0.005**
	V3	10.5 (22.2)	2.1 (0.0, 12.6)		31.5 (61.3)	3.2 (1.0,33.6)		47.8 (78.0)	10.7 (1.3, 75.6)	
Entero-bacteriaceae	V0	12.8 (19.6)	0.0 (0.0, 21.0)	0.195	8.5 (16.4)	0.0 (0.0, 9.4)	0.559	8.55 (17.2)	0.0 (0.0, 10.5)	0.057
	V3	7.0 (10.7)	2.1 (0.4, 8.9)		3.3 (6.3)	0.7 (0.2, 3.1)		5.9 (13.3)	0.5 (0.0, 6.1)	

Cfu: colony-forming unit.

5.3.11. Multivariate analyses on the effects of SYN1 supplementation on infant's stools frequency and consistency

Linear regression analyses with two covariates (i.e., formula and bacterial strain) were performed to assess the effect of microbiota in frequency and consistency score (Table 39). This analysis revealed a significant direct association of the SYN1-supplemented formula ($p<0.05$) and the level of gut Bifidobacterium ($p<0.01$) with the faecal consistency score, explaining up to 13.5% of its variability.

In a second linear regression model we found a direct association of the SYN1 formula ($p<0.05$) and an inverse association of Enterobacteriaceae counts in the gut ($p<0.001$) with the stools frequency. The variability of the stool frequency was explained up to 18.2% by both the study formula and Enterobacteriaceae levels.

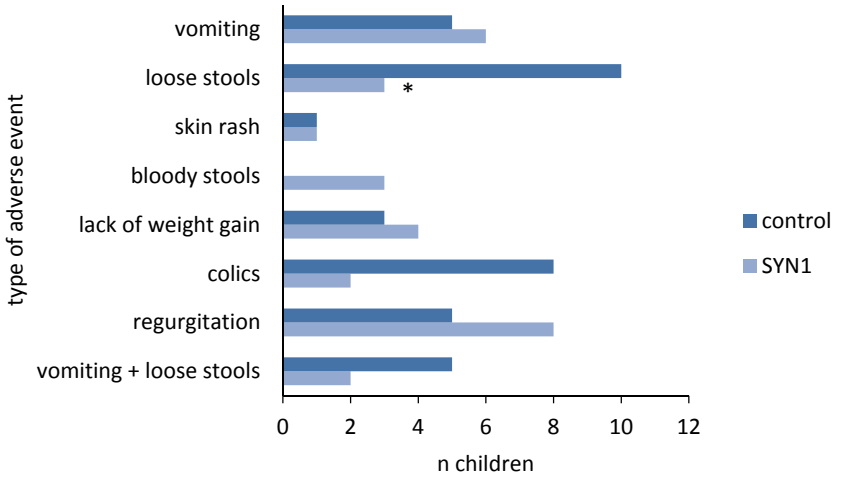
Table 39. Linear regression analyses for variables with effect on faecal consistency score and stools frequency.

	β	Confidence interval 95% (min, max)	p-value	R^2
Variables with effect on faecal consistency score				
Type of formula (SYN1/Control)	0.200	(0.022, 0.690)	**0.037**	0.135
Bifidobacterium (log)	0.296	(0.054, 0.243)	**0.002**	
Variables with effect on stools frequency				
Type of formula (SYN1/Control)	0.516	(0.045, 0.987)	**0.032**	0.182
Enterobacteriaceae (log)	-0.347	(-0.521, -0.173)	**0.000**	

5.3.12. Adverse events

A total number of 66 possible adverse events were reported by parents during the whole study period and they are plotted in Figure 14. They were equally occurring in both formula groups apart from slightly less loose stools in the SYN1 group.

Figure 14. Adverse events (possible related to the study product or not) reported during all the study period.



* p<0.05 vs. control group

Three infants within the SYN1 group developed transiently bloody stools. These episodes were likely related to some transient lactose or cow's milk intolerance without clinical relevance. None of these infants were lactose or cow's milk intolerant at 1 year old (follow-up medical check).

5.3.13. Intention to treat analyses (ITT)

The results of the ITT analyses (appendix 9.4) did not show any significant difference on anthropometrical data between formula groups at any time point and throughout the study.

No significant differences were found between the two formula groups in any urinary parameter at any timepoint. Furthermore, the concentrations of these parameters were in the SYN1 group always closer to the breastfed group than in the control group.

The safety serum parameters like minerals (Na, K, Cl, Ca-ion), pH and proteins (total protein, albumin, pre-albumin) were comparable between the formula groups, apart from urea, which concentration was slightly higher with standard formula.

ITT analyses also confirmed significant differences between formula groups in number of depositions and faecal consistency score at all study timepoints and throughout the study (data from the parents' diary). The SYN1 supplemented infants had more depositions and softer stools.

Regarding formula tolerance, there were no significant differences in vomits and regurgitations between the two formula groups at all time points, except at month 4 for vomits that can be fully attributed to the statistical procedure (medians and IQR all 0) rather than to a biological meaning.

And, finally, consistently with results from parents' diaries, there were no differences between formula groups in the recall of mother's perception of infant's frequency of digestive discomfort, frequency of vomits or frequency of regurgitation at any of the timepoints and throughout the study.

DISCUSSION

6. DISCUSSION

This is the first randomized clinical trial to comprehensively demonstrate the safety, tolerance and efficacy of a 0.8g/dl SYN-1-supplemented infant formula for healthy, term infants during the first 4 months of life. Although a previous study assessed the effects of SYN1 on newborns, the study period was only four weeks, and the water balance was not assessed. Our study extended the observation time to assess whether the softer and more frequent stools that were observed affected the infants' growth or hydration status.

6.1. EFFICACY

The efficacy of the SYN1-supplemented formula was demonstrated by both the microflora analyses and the improved stools patterns.

The composition of the microflora differed between the two groups, having the inulin-oligofructose mixture a bifidogenic effect. The infants who were supplemented with 0.8 g/dL SYN1 showed a trend toward increased total counts of *Bifidobacterium* in the faecal flora when compared with the infants who were fed a non-supplemented formula, however differences were only borderline significant. The *a posteriori* calculation of statistical power revealed that we would need 87 children per feeding group to find out statistically significant differences about 11% in log cfu/g faeces of *Bifidobacterium*. Although differences in the *Bifidobacterium* count were only borderline significant between feeding groups, the proportion of *Bifidobacterium* among the whole microbiota

confirm the predominance of this bacteria in the gut of infants supplemented with SYN1 compared to control infants (Figure 13). Similar results were reported by Veereman-Wauters et al., who observed significant higher *Bifidobacterium* counts at day 14 in infants fed with SYN1 0.8 g/dL and FOS (long-chain inulin):GOS formulas compared with the SYN1 0.4 g/dL and control groups. Also, in the SYN1 0.8 g/dL and FOS (long-chain inulin):GOS groups, *Bifidobacterium* counts were significantly higher at day 14 and 28 compared with day 3, and were comparable with the breast-fed group (129). In our study, this positive effect persisted for at least 3 months. In contrast, other authors were unable to find significant differences in *Bifidobacterium* in infants fed prebiotic or standard formulas after 6 weeks of intervention (120). Another study supported these results, observed that at the end of 28-day feeding period, the *Bifidobacteria* counts was significantly higher in supplemented formulas with FOS:GOS versus control formula without supplementation (119).

The stools pattern of the SYN1-supplemented infants was characterized by a higher number of depositions and a softer consistency throughout the study period when compared with the non-supplemented infants (Figure 11 and Figure 12). Furthermore, ITT analyses confirm this effect on frequency and consistency stools. This pattern was more similar to the pattern in breastfed infants. The softer and more frequent stools observed in the SYN1-supplemented group may partially be explained by the higher *Bifidobacterium* colonization noted previously, as shown by the linear regression analyses. These results are consistent with Costalos et al. who

found a higher stool frequency and significant softer stools in prebiotic group compared to the control group (120).

This improvement of stools frequency and consistency is of great interest, given that this may partly reduce the adverse effects (i.e., constipation) of feeding infants a standard formula rather than breastfeeding (154). Tunc VT, et al. showed a hard stools deposition in 1.1% of breastfed infants and in 9.2% of formula fed infants (p=0.001) (122).

Regarding the immune system, Van Stuijvenberg M, et al. (155) showed that there was no difference in the number of fever episodes between the prebiotic formula (FOS/GOS) and control formula, neither during the intervention (6 months) nor later on (12 months). Our results are consistent with those from Van Stuijvenberg M et al. and do not contradict those from Arslanoglu et al. In this randomized clinical trial, Arslanoglu et al. (73) did not find any significant difference during the first 4 months of life between infants fed supplemented or not with a FOS/GOS infant formula. However, they found lower infections incidence from the 4th to 6th month of life. Possibly, we hypothesize that a longer-term follow-up of the infants from our study would help to elucidate the possibility of preventing infections by prebiotic supplementation in infants.

6.2. SAFETY AND TOLERANCE

The prebiotics' fermentation process in the intestine may be associated with the onset of gastrointestinal symptoms (e.g., vomiting, regurgitation,

diarrhoea or flatulence) and irritability (e.g., agitation and crying). The presence of these symptoms could lead to the discontinuation of prebiotic intake or could interfere with the child's growth. Consequently, we assessed whether the formula with prebiotics influenced the overall intake of milk and calories. We noted similar formula intakes and acceptance in the two groups of infants. Thus, the study results suggest that SYN-1 supplementation is safe in terms of infant ingestion. Some authors have reported a similar intake for the standard and prebiotic-supplemented formulas in infants (119;129), whereas others have not assessed this parameter (118).

A major concern was evaluating the infants' growth during the first months of life. All of the infants exhibited similar growth patterns. The infants fed with the prebiotic formula experienced similar weight, length and head circumference gains during the first four months of life, and the fatty body composition assessed by skinfolds was similar for both formula groups. These results were confirmed with the ITT analyses, which also showed similar growth patterns. Although two different meta-analyses have shown that prebiotic supplementation is associated with slightly greater weight gains (71;131), some previous studies have reported no significant differences in growth in preterm and term infants fed with prebiotic-supplemented formulas (119;129;156). Moreover, a Cochrane Review concluded that the prebiotic supplementation of infant formula had no negative effects on infant growth (71). A recent study of infants who consumed prebiotics during the first year of life revealed no differences in growth during this long follow-up period (157). Accordingly, the most recent systematic review and commentary by the ESPGHAN

Committee on Nutrition concluded that the supplementation of infant formula with prebiotics has no adverse effects on growth in healthy, term infants (132).

In our clinical trial, the infant formula had no relation on the subjects' reasons for dropping out the study. The digestive symptoms (e.g., regurgitation or gastrointestinal symptoms) reported as reasons to withdraw the study were those that are characteristic of this period of infancy (for both formula- and breastfed infants). Typical gastrointestinal symptoms (e.g., regurgitation, vomiting and digestive discomfort) and crying behaviour during the follow-up period were comparable between the formula groups. Similar results have been reported by other authors (119;129).

The arms analyses have shown as well, that there was not increased proportion of adverse events within the SYN1 supplemented group.

The SYN1 supplementation group had softer and more frequent stools, which could be regarded as an indicator of positive gut health and was not associated with an impaired water balance. Although other authors have reported similar results, none of them have assessed the possible side effects of having softer and more frequent stools (119;120;129). It is important considering that increased loose and watery stools in infants at this early age range could lead to adverse events such as dehydration. Therefore, it is worth stressing that the increases in the number of depositions and in stools softness were not accompanied by changes in the hydration status, as demonstrated by the water balance analyses. Both the osmolarity of the urine and the sodium-potassium ratio were similar between formula-fed groups, reflecting comparable hydration

levels. Both formula groups also had similar urinary concentrations of ions and creatinine, with the exception of K at month 1, which was slightly lower in the SYN1-supplemented group and was more similar to that of the breastfed infants than that of the control group. Moreover, these results were supported by the ITT, which showed no significant differences between feeding groups. These results have special relevance because, to the best of our knowledge, none of the previously published trials assessing the effects of prebiotic supplementation in infant formulas have measured this safety parameter.

In addition to the urinary determinations, we also measured the blood serum parameters to assess safety (Table 22). The plasmatic ion and bicarbonate concentrations and pH values were similar in both of the formula-fed groups, showing no electrolyte abnormalities. The cholesterol plasmatic levels were similar for both formula groups. Additionally, both formula groups had similar blood protein patterns, with parallel levels of total proteins, albumin and prealbumin. The urea concentrations of the SYN1-supplemented infants were lower than those of the control group and more similar to those of the breastfed group. Few studies of infants fed with prebiotic-supplemented formulas have measured blood parameters. Alliet et al. identified no differences in lipid parameters (i.e., total cholesterol) between control and prebiotic-supplemented formula groups (158), whereas others have noted no differences in total proteins, albumin and urea and only slight differences in prealbumin between control and prebiotic-supplemented formula groups (124).

Considering all these analyses, in which there is no effect on blood and urinary parameters, we can conclude that the SYN1 supplementation through infant formula is safe.

A limitation of this study may be the relative high dropouts' rate. However, we believe that this high withdrawals proportion is usual in this kind of nutritional clinical trials in healthy infants, where colics and crying behaviour make families changing formulas. Therefore, we checked that reasons to drop out were similar in both formula groups during the whole study period. Finally the intention to treat analyses performed confirm that our results were not biased for the high drop outs rate.

Another possible limitation could be that infants could have been recruited during the first 4 weeks of life (being influenced by other type of feeding). However, as we did not find any difference at inclusion age between feeding groups and we did not find differences in distribution of infants recruited after hospital discharge, we think that the final results of our primary outcomes have not been affected.

The main strengths of this study are its clinical trial design, controlling all factors that may be related to safety and efficacy, the adherence to CONSORT statements (as the adverse events and drop outs records) and the intention to treat analyses performed, that provide high robustness to our results.

It was already proposed that 0.8g/dL of SYN1 in formula milk might be a more effective dose than 0.4g/dL of SYN1 and similar to FOS/GOS supplementation (129). Our clinical trial demonstrates the safety and efficacy of this SYN1 0.8g/dL supplemented formula. From a practical

point of view, this clinical trial may support the use of this prebiotic in infant formulas.

In summary, this study demonstrates that SYN1 supplementation promotes a trend toward increased *Bifidobacterium* in the gut which might be the physiological mechanism to promote a deposition pattern closer to that promoted by human milk. Furthermore this softer stools pattern was not associated with any harmful effect such as disturbed growth or water balance. We conclude that 0.8 g/dL SYN1 supplementation in infant formula during the first 4 months of life is safe, effective and well tolerated.

CONCLUSIONS

7. CONCLUSIONS

Tolerance:

- There were no differences in the digestive tolerance of SYN1 supplemented formula during the first four months of life.
- There were no differences in the quantity of formula ingested between the supplemented or non-supplemented group.

Safety:

- The growth was similar and there were no differences in anthropometry between supplemented formula SYN1 infants and the control formula infants, during the first four months of life.
- There were no relevant differences in the water-electrolyte balance, proteins, minerals and kidney function in infants fed with the supplemented formula or placebo formula, during the first three months of life.

Efficacy:

- Infants with SYN1 formula had significantly higher stool frequency at all timepoints compared to the control group. Stool consistency was significantly softer in SYN1 group, being closer to the breastfed group in all timepoints.
- A possible mechanism to modulate the intestinal microflora in the SYN1 formula group was through Bifidobacteria. It was significantly higher and cell counts were by trend higher in the SYN1 group compared to the control group.

- The incidence of illness, atopic dermatitis and serum concentration of immunoglobulin were similar between formula groups.

The results demonstrate that infant formula supplemented with 0.8g/100mL of SYN1 is safe, well tolerated and effective.

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ADDENDUMS

9. ADDENDUM

9.1. ADVERSE EVENTS

Table 1. Adverse event form to complete by researcher

IDENTIFICATION NUMBER:

	Adverse effect 1	**Adverse effect 2**	**Adverse effect 3**
Report date			
Event(s)			
Severity (If there has been any of the following cases, the effect has been severe)	YES Death ☐ death risk ☐ permanent inability ☐ prolonged hospitalization ☐ medically relevant ☐ No ☐	YES Death ☐ death risk ☐ permanent inability ☐ prolonged hospitalization ☐ medically relevant ☐ No ☐	YES Death ☐ death risk ☐ permanent inability ☐ prolonged hospitalization ☐ medically relevant ☐ No ☐
Date of 1st appearance			
Duration	_____hours _____days	_____hours _____days	_____hours _____days
Frequency	once ☐ several ☐ remains ☐	once ☐ several ☐ remains ☐	once ☐ several ☐ remains ☐
Intensity	Mild (1) ☐ moderate (2) ☐ intense (3) ☐	Mild (1) ☐ moderate (2) ☐ intense (3) ☐	Mild (1) ☐ moderate (2) ☐ intense (3) ☐
The adverse effect, cause to stop the study?	Yes ☐ No ☐	Yes ☐ No ☐	Yes ☐ No ☐

Has the AE caused the infant formula abandonment?	YES ☐ NO ☐	YES ☐ NO ☐	YES ☐ NO ☐
If yes:			
Has the AE disappeared?	YES ☐ NO ☐	YES ☐ NO ☐	YES ☐ NO ☐
Has the study product been reintroduced?	YES ☐ NO ☐	YES ☐ NO ☐	YES ☐ NO ☐
If yes:			
Has the AE reappeared?	YES ☐ NO ☐	YES ☐ NO ☐	YES ☐ NO ☐
If yes: date of last appearance			
If yes: Result	AE disappeared ☐ AE remains ☐ Unknown ☐	AE disappeared ☐ AE remains ☐ Unknown ☐	AE disappeared ☐ AE remains ☐ Unknown ☐
Had the infant suffered previously this AE?	YES ☐ NO ☐ Unknown ☐	YES ☐ NO ☐ Unknown ☐	YES ☐ NO ☐ Unknown ☐
AE treatment	YES ☐ NO ☐ Specify	YES ☐ NO ☐ Specify	YES ☐ NO ☐ Specify
Causality (AE-assay)	Unrelated ☐ unlikely ☐ Likely ☐ Certainly ☐	Unrelated ☐ unlikely ☐ Likely ☐ Certainly ☐	Unrelated ☐ unlikely ☐ Likely ☐ Certainly ☐

9.2. VISIT FORMS / MEDICAL HISTORY / PHONE CALLS

INCLUSION FORM

Initials:_____ Date of birth:___/____/_____ Gender: □ male □ female

Inclusion criteria	**Yes**	**No**
Healthy	□	□
Term infant (37-42 weeks)	□	□
Normal birth	□	□
AGA (3rd to 97th percentiles)	□	□
Normal feeding behaviour	□	□
≥90 % of energy intake fed by human milk or by infant	□	□
Baby fulfils all inclusion criteria?	□	□

Exclusion criteria	**Yes**	**No**
Age > 4w-old	□	□
Serious respiratory, neurological, gastrointestinal or metabolic disorders	□	□
Infections or other serious diseases*	□	□
Use of a therapeutic formulae (>10% of total intake),	□	□
Parents or guardians cannot be expected to comply with	□	□
Parents or guardians have not command on Catalan or Spanish language	□	□
Baby fulfils any of the exclusion criteria?	□	□

** That could interfere in normal feeding or growth*

If baby fulfils all inclusion criteria and doesn't fulfil any of the exclusion criteria, parents can be asked to sign written consent.

Do parents accept to sign written consent? □ Yes □No

V0 Birth	Child Initials |___| ID |___| Random|____|	DATE ___/___/_____

BASELINE DATA

1. Date of birth: _____ / _____ / _____

2. Gender: □ Male □ Female 3. Parity ___ / ___ / ___ / ___

4. Gestational age: weeks____Days:_____ 5. APGAR score (1-10'): _____

Anthropometry at birth

6. Weight: __ ___ g 7. Length:_____cm 8. Head circumference: __cm

9. Antibiotic intake by mother during 4 wks prior to delivery:

□ No □ Yes, Name_____ Duration_____

specify

10. Antibiotic administration to baby:

□ No □ Yes, Name_____ *If yes, baby could not be included in the study*

specify

11. Way of delivery: □ Vaginal □ C-section

12. Were there complications in the course of pregnancy?

□ No □ Yes, specify:_____

□ Gestational diabetes □ Hydramnios

□ Hypertension □ Oligohydramnios

□ Pre-eclampsia/ Eclampsia □ Others: specify

□ Bleeding in pregnancy _____

13. Did the child's mother take any medication during pregnancy? □ No □ Yes, specify:

□ Insulin □ Corticosteroids

□ Oral antidiabetics
□ Beta-blockers
□ Thyroid hormones, antithyroid drugs
□ Antidepressives
□ Anxiouslithic
□ Others _____

14. Did the mother experience stress during pregnancy

□	□	□	□	□
Not at all	Rarely	Sometimes	Frequently	All the time

15. During pregnancy, stress was arose due to

□ Occupational strain problems
□ Financial problems
□ Lack of support by partner/ child's father
□ Lack of support by the social milieu /family other than partner
□ Health problems related to pregnancy
□ Health problems no related to pregnancy
□ Other _____
□ Refused to say

16. The pregnancy was:

□ Planned
□ Not planned, though the parents wished to have a child at some time
□ Not planned, the parents did not wish to have a child at that time
□ Refused

17. Is there family history of allergy? Yes □ No □

	Atopic dermatitis	Asthma	Food allergy	Others, specify
Mother	□	□	□	□
Father	□	□	□	□
1st Sibling	□	□	□	□
1st Sibling	□	□	□	□
Other: specify	□	□	□	□

V0 Birth	Child Initials |___| ID |___| Random|____|	DATE ___/___/_____

SOCIO-DEMOGRAPHIC QUESTIONNAIRE

1. Which is the mother's nationality?

   □ Spanish □ Other => *Please specify*_____

2. Which is the father's nationality?

   □ Spanish □ Other => *Please specify*_____

3. Maternal command of the national language

   □ Mother language
   □ Excellent
   □ Good
   □ Moderate
   □ Poor
   □ Does not speak the national language
   □

4. Residence in the study area (at inclusion)

   □ Yes
   □ No

5. Parents' date of birth:

Mother: |_____|_____|_____| Father : |_____|_____|_____|

month day year **month day year**

6. Marital status

   □ Married or unmarried, lives together with the baby's father
   □ Single mother
   □ Widowed
   □ Refused to say

7. Number of adults (at least 18 years) living in the household |____|

8. Number of children including study child (below 18 years) living in the household |_____|

How many older siblings has the child got (including half-brothers and sisters)?
(If he or she hasn't got any older siblings, please enter 0.)

Number of older siblings |_____|

9. What is the highest level of education the child's mother/father has completed (according to the International Standard Classification of Education - ISCED-97).

	Mother	Father
ISCED 0 – Pre-preliminary level of education	☐	☐
ISCED 1 – Primary level of education	☐	☐
ISCED 2 – Lower secondary level of education	☐	☐
ISCED 3 – Upper secondary level of education	☐	☐
ISCED 4 – Post-secondary non-tertiary	☐	☐
ISCED 5 – First stage of tertiary education	☐	☐
ISCED 6 – Second stage of tertiary education	☐	☐
No schooling completed	☐	☐
Unknown	☐	☐

(see Manual for ISCED-97 Implementation in OECD Countries – 1999 edition)

10. Last week, was the **child's mother** doing any work:
    - as an employee, or on a Government sponsored training scheme
    - as a self-employed person/ freelance, or in the her/his own/family business

    'Yes' if away from work ill, on maternity leave, on holiday or temporarily laid off.

'Yes' for any paid work, including casual or temporary work, even if only for one hour.

'Yes' if she worked paid or unpaid in her own/family business

□ Yes *If Yes please continue with question!*

□ No *If No please continue with question*

11. Was the child's mother actively looking for any kind of paid work during the last 4 weeks?

□ Yes □ No

12. Last week, was the child's mother any of the following: *Tick all the boxes that apply.*

□ Student

□ Looking after home/family

□ Permanently sick/disabled

□ Retired

□ None of the above

13. Has the child's mother ever worked?

□ Yes, please write in the year she last worked |__|__|__|__|

□ No, she has never worked *=> If No, please continue with question 17!*

The questions 14 -16 should be answered for the main job the child's mother was doing last week, or if not working last week, her last main job. The main job is the job in which she usually works for the majority of working hours.

14. Does (Did) she work as an employee or is (was) she self-employed?

□ Employee

□ Self-employed/freelance with employees

□ Self-employed/freelance without employees

15. How many hours a week does (did) she usually work in her main job?

Answer to nearest whole hour and give average for last four weeks

Number of hours worked a week |__|__|

16. What is (was) the full title of her main job?

For example primary school teacher, sales assistant, engineer. Give job titles not grade or pay band

_____

_____

17. Last week, was the **child's father** doing any work:

- as an employee, or on a Government sponsored training scheme
- as self-employed/freelance, or in the her/his own/family business

'Yes' if away from work ill, on paternity leave, on holiday or temporarily laid off.

'Yes' for any paid work, including casual or temporary work, even if only for one hour.

'Yes' if he worked paid or unpaid in his own/family business

□ Yes *If Yes please continue with question 21!*

□ No *If No please continue with question!*

18. Was the child's father actively looking for any kind of paid work during the last 4 weeks?

□ Yes □ No

19. Last week, was the child's father any of the following:

Tick all the boxes that apply.

□ Student

□ Looking after home/family

□ Permanently sick/disabled

□ Retired

□ None of the above

20. Has he ever worked?

□ Yes, please write in the year he last worked |__|__|__|__|

□ No, he has never worked *If No, the questionnaire has been finished.*

The questions 21 - 23 should be answered for the main job the child's father was doing last week, or if not working last week, his last main job. The main job is the job in which she usually works for the majority of working hours.

21. Does (Did) he work as an employee or is (was) he self-employed?

□ Employee

□ Self-employed/freelance with employees

□ Self-employed/freelance without employees

22. How many hours a week does (did) he usually work in his main job?

Answer to nearest whole hour and give average for last four weeks

Number of hours worked a week |__|__|

23. What is (was) the full title of his main job?

For example primary school teacher, car mechanic, television service engineer.

Give job titles not grade or pay band

_____

_____

T1 0.5 month	Child Initials |___| ID |___| Random|____|	DATE ___/___/_____

TELEPHONE INTERVIEW CHECKLIST

1. Which type of milk do you feed?

	when **started**	when **stopped**	**still continuing**
O breast milk	week _____	week _____	**O**
O study formula	week _____	week _____	**O**
O other formula (*)	week _____	week _____	**O**

(*) if has been fed >10%, infant should be excluded from the study.

2. If you feed the study formula, how does your child accept the formula.....?

O	**O**	**O**	**O**	**O**
very good	good	moderate	poor	very poor

3. Does your child get any supplements on a regular basis (3 or more times a week)? Please tell us what kind of supplements your child gets, the name and the manufacturer of the preparation and the dose/amount your child takes per day!

O no **O** yes, which:

vitamins:..... ..... quantity/day:.....

minerals:.....quantity/day:.....

other:.....quantity/day:.....

4. Does the mother get any supplement? (Answer only if the child is breastfed)

O no **O** yes, which:

vitamins:..... quantity/day:.....

minerals:.....quantity/day:.....

other:.....quantity/day:.....

V1 1 month	Child Initials |___| ID |___| Random|____|	DATE ___/___/_____

INFANT'S BEHAVIUOR

17. How often does the child cry on average and how long do these periods take approximately?

|__| times during the night (ca. 7 p.m. – 6 a.m.) |____| h |____| min

|__| times during the daytime (ca. 6 a.m. – 7 p.m.) |____| h |____| min

18. How long does the child cry on average?

during the night (ca. 7 p.m. – 6 a.m.) |____| h |____| min
during the daytime (ca. 6 a.m. – 7 p.m.) |____| h |____| min

19. Does the child ever cry or whine **for at least 3h/ day**? □ Yes □ No

20. Does this happen on **at least 3 days in any week**? □ Yes □ No

21. Did this crying/whining last for **at least 3 weeks**? □ Yes □ No

22. How old was the child, when this crying/ whining started? |____| days

23. How much do you think your child cries/whines?

□	□	□	□	□
Not enough	Average	Above average	A lot above average	I can't tell

24. Do you feel stressed by the child's crying/whining?

□	□	□	□	□
Not at all	Mildly	Moderately	Strongly	Very strongly

25. When the child cries or whines, how hard is it to calm him/her down?

□	□	□	□	□
Very easy	Easy	Moderate	Difficult	Impossible

If not *'Impossible'*, how do you succeed in calming the child?
□ Pacifier/dummy
□ Rocking to sleep
□ Breastfeeding
□ Giving the bottle
□ Other *Please specify*_____

V1 1 month	Child Initials |___| ID |___| Random|____|	DATE ___/___/_____

ANTHROPOMETRY

	1st measure	***2nd measure***	***3rd measure***
1 - Weighing Scale			
Weight	|__|__|,|__|__|kg	|__|__|,|__|__|kg	
2 - Tape measures			
Head circumferenc	|__|__|,|__|cm	|__|__|,|__|cm	
Waist circumference	|__|__|,|__|cm	|__|__|,|__|cm	
(WHO) recumbent position			
Mid upper arm circumference	|__|__|,|__|cm	|__|__|,|__|cm	
3 - Skinfold calliper			
Triceps skinfold	|_|_|,|_|m	|_|_|,|_|mm	|_|_|,|_|mm
Subscapular skinfold	|_|_|,|_|m	|_|_|,|_|mm	|_|_|,|_|mm
6 - Infant measuring table			
Recumbent length	|__|__|__|,|__|cm	|__|__|__|,|__|cm	

V1 1 month	Child Initials |___| ID |___| Random|____|	DATE ___/___/_____

MEDICAL HISTORY / DIGESTIVE SYPTOMS

Vaccination: □ No □Yes

Name:_____ Date: ___/___/_____

Allergy: atopic dermatitis □ No □Yes

Digestive symptoms:

Digestive discomfort: □ yes □ no

Vomiting: □ yes □ no

Regurgitation: □ yes □ no

Stool frequency: _____times _____day // _____times _____ week

Stool consistency: type_____ (1 to 5 from Bristol scale)

Feeding type:	**Yes**	**No**
Breast feeding > 90%	□	□
Study formula > 90 %	□	□
Other formula >10% (if >10%, infant should be excluded from the study).	□	□

Illness and medication:

Child illness episodes:			
Age (weeks)	Diagnose	Medication	Duration (days)

If breastfed, mother's drugs: No □ Yes □ Name_____ ____Duration _____days

V1 0- 1 month	Child Initials |___| ID |___| Random|____|	DATE ___/___/_____

CASE REPORT FORM

Has your baby been vaccinated? No Yes

Name:_____ Date:___ / ___ / ___

EVENTS ALONG 4 WEEKS:

Child illness episodes:			
Age	Diagnose	Medication	Duration (days)

Complete only if breastfed:

If breastfed, mother's Mother illness episodes:			
Age	Diagnose	Medication	Duration (days)

V1 1 month	Child Initials |___| ID |___| Random|____|	DATE ___/___/_____

FEEDING HABITS AND BEHAVIOUR

Date: |_____|_____|_____| (day 1)
month day year

1. Is your child uneasy, excited or does your child cry or whine during the meals, so that this behaviour makes the feeding very difficult?

O	**O**	**O**	**O**	**O**	**O**
never	rarely	sometimes	frequently	always	I can't tell

2. Do you have to distract your child in order to feed him or her?

O	**O**	**O**	**O**	**O**	**O**
never	rarely	sometimes	frequently	always	I can't tell

3. How would you assess your child's appetite?

O	**O**	**O**	**O**	**O**	**O**
poor	moderate	normal	very good	excessive	I can't tell

4. Do you feel, that the amount of food consumed by your child is:

O	**O**	**O**	**O**	**O**	**O**
too low	low	enough	adequate	excessive	I can't tell

5. Do you feel stressed by the eating behaviour of your child?

O	**O**	**O**	**O**	**O**	**O**
severely	strongly	moderately	mildly	not at all	I can't tell

6. Have you already started introducing solid foods (complementary foods)?

O no **O** yes, from which week on? .....week

7. How often does the child cry **on average** and how long do these periods take **approximately**?

|__| times during the night (ca. 7 p.m. – 6 a.m.)|_____| h |____| min

|__| times during the daytime (ca. 6 a.m. – 7 p.m.)|_____| h |____| min

8. How long does the child cry **on average**?

during the night (ca. 7 p.m. – 6 a.m.) |_____| h |_____| min

during the daytime (ca. 6 a.m. – 7 p |_____| h |_____| min

3. Does the child ever cry or whine **for at least 3 hours a day**?

O Yes **O** No ⇒ Please continue with question!

4. Does this happen on **at least 3 days in any week**?

O Yes **O** No ⇒ Please continue with question!

5. Did this crying/whining last for **at least 3 weeks**?

O Yes **O** No ⇒ Please continue with question!

6. How old was the child, when this crying/whining started? |____| days

7. How much do you think your child cries/whines?

O	**O**	**O**	**O**	**O**	**O**
Not at all	Not to	Average	Above	A lot above	I can't tell

8. Do you feel stressed by the child's crying/whining?

O	**O**	**O**	**O**	**O**	**O**
Not at all	Mildly	Moderately	Strongly	Very	I can't tell

9. When the child cries or whines, how hard is it to calm him/her down?

O	**O**	**O**	**O**	**O**	**O**
Not at all	Mildly	Moderately	Strongly	Very	I can't tell

If not *'Impossible'*, how do you succeed in calming the child?

O Pacifier/dummy

O Rocking to sleep

O Breastfeeding

O Giving the bottle

O Other Please specify_____

INSTRUCTIONS TO COMPLET THE "PARENT'S DIARY"

Dear Participant!

Please, fill in the case report, registering any illness event that your baby could undergo during these 4 weeks. Also, medications and vaccination details should be recorded if it is the case.

On other hand, fill in the diary at the end of the second week after the visit. The diary contains different parts:

1. The "FEEDING HABITS AND BEHAVIOUR" questionnaire.

2. The "DIGESTIVE SYMPTOMS" and "DIETARY INTAKE" diary:

⇒ **Digestive symptoms**, it should be completed a table with the number of depositions, type of depositions (looking at the Bristol scale figures, attached below), number of regurgitations and number of vomits

What is exactly regurgitation? To throw out through mouth, without vomiting, solid or liquid substances contained in the stomach.

What is exactly vomiting? To bring up through mouth solid or liquid substances*, with some force, by retch.*

⇒ **Dietary intake diary**,

To assess the eating habits of your child, it's necessary to conduct a food record over 2 consecutive days. It's important to accurately record everything that your child has eaten!

If it is not possible at certain times for you to fill in this form, please ask the person who takes care of your child at that time (i.e. grand-mother, day-care attendant) to record the exact amounts of milk meals or other foods consumed by the child.

How to fill out the record properly?

- → Please record breast-feeding and/ or the intake of formula or other consumed fluids (like tea, juices… if it is the case) in the provided charts.
- → Note immediately the exact time when your child has a milk meal or drinks anything else.
- → Please, note that it is understood a complete day **from 00h to 24h.**

- → Formula intake:
  - Note type of the formula (ie: study formula).
  - Please record the type, brand and amount of other ingredients which you have been used for bottle preparation (if it is the case). Give the number of tea- or tablespoons, grams or millilitres.
  - Note the amount of milk that has been offered to your child in the bottle and also the volume your child has drunk at each meal.

- → Other fluid intake:
  - Please record the type, the brand and the amount of fluid consumed (e.g. apple juice, brand, 50 ml).
  - Note the age of your child, when it was given a certain fluid for the first time.

V1 1 month	Child Initials |___| ID |___| Random|____|	DATE ___/___/_____

DIGESTIVE SYMPTOMS DIARY

Date: |_____|_____|_____| (**day 1**) **(from 00h to 24h)**

month day year

DAY PERIOD	**NUMBER OF DEPOSTIONS**	**DEPOSITION TYPE (Bristol scale) ***	**REGURGITATION (number of episodes)**	**VOMITING (number of episodes)**
From 0h to 4h				
From 4h to 8h				
From 8h to 12h				
From 12h to 16h				
From 16h to 20h				
From 20h to 24h				

Food record at the end of 4 weeks (<u>day 1</u>)

Date |_____|_____|_____| (**<u>day 1</u>**) **(from 00h to 24h)**

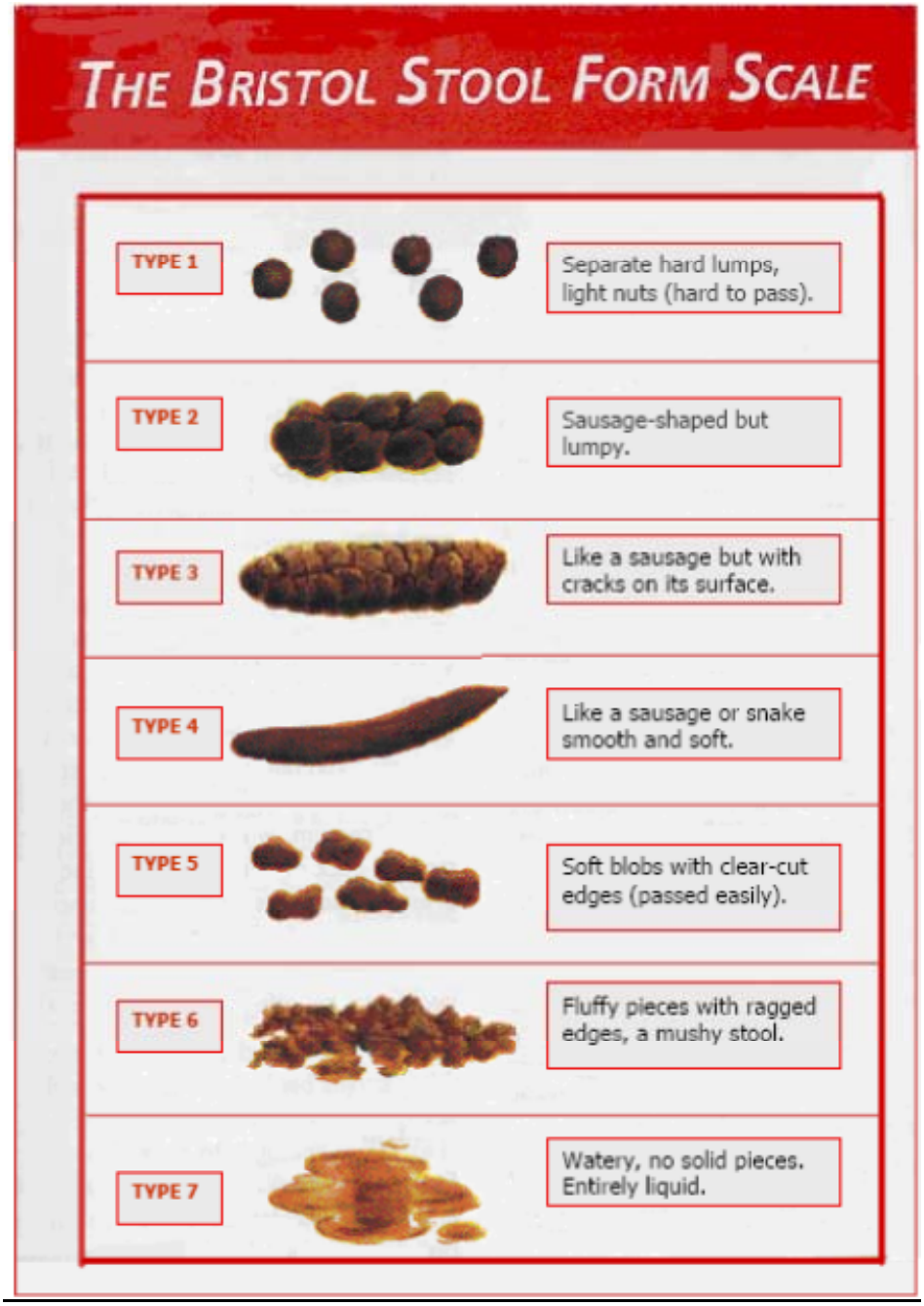
MILK INTAKE					
Time	Type of milk	Amount of formula prepared	Other		Remaining volume after meal
		(ml)	(type, brand)	(g, ml or spoons)	(ml)

OTHER FLUID INTAKE (juice, …)	
Type of fluid	Volume consumed/ day
(type, brand)	(ml)

PREPARATION OF THE BOTTLE:

Please, explain how you prepare the bottles for your baby (amount of each ingredient).

COMMENTS:
Please, don't forget to detail any comment about your baby's intake: special additions to bottle, special preparation, new fluids introduced in his/ her diet (tea, juice...).


THE BRISTOL STOOL FORM SCALE
TYPE 1
Separate hard lumps, light nuts (hard to pass).
TYPE 2
Sausage-shaped but lumpy.
TYPE 3
Like a sausage but with cracks on its surface.
TYPE 4
Like a sausage or snake smooth and soft.
TYPE 5
Soft blobs with clear-cut edges (passed easily).
TYPE 6
Fluffy pieces with ragged edges, a mushy stool.
TYPE 7
Watery, no solid pieces. Entirely liquid.

V1 1 month	Child Initials |___| ID |___| Random|____|	DATE ___/___/_____

DIGESTIVE SYMPTOMS DIARY

Date: |_____|_____|_____| (**day 2**) **(from 00h to 24h)**

month day year

DAY PERIOD	**NUMBER OF DEPOSTIONS**	**DEPOSITION TYPE (Bristol scale) ***	**REGURGITATION (number of episodes)**	**VOMITING (number of episodes)**
From 0h to 4h				
From 4h to 8h				
From 8h to 12h				
From 12h to 16h				
From 16h to 20h				
From 20h to 24h				

Food record at the end of 4 weeks (<u>day 2</u>)

Date |_____|_____|_____| (**<u>day 2</u>**) **(from 00h to 24h)**

MILK INTAKE					
Time	Type of milk	Amount of formula prepared	Other		Remaining volume after meal
		(ml)	(type, brand)	(g, ml or spoons)	(ml)

OTHER FLUID INTAKE (juice, …)	
Type of fluid	Volume consumed/ day
(type, brand)	(ml)

PREPARATION OF THE BOTTLE:

Please, explain how you prepare the bottles for your baby (amount of each ingredient).

COMMENTS:
Please, don't forget to detail any comment about your baby's intake: special additions to bottle, special preparation, new fluids introduced in his/ her diet (tea, juice...).

9.3. WRITTEN INFORMED CONSENT

WRITTEN CONSENT

Mss……informs to Mr./Mrs…… about the development of the inulin-oligofructose infant study.

Research team declares:

The aim of this project is to demonstrate the safety and efficacy of an infant formula supplemented with a mixture of inulin and oligofructose, including appropriate infants' growth. Additionally, effects on immunity, modulation of the composition of the gut microflora, and gut functioning will be studied and compared to a non-supplemented infant formula, as well as to compare these parameters with breast-fed infants (reference group).

Research team guaranties the confidentiality on participants' identity and also guaranties that blood samples and derived results will be used for the detailed objectives of the study, and not for other purposes.

Mother / Father declares:

- It has been given to me the parents' information sheet, and has been informed about the study summarized in this sheet.
- I have been able to do questions to clarify my doubts.
- It is guaranteed that health attention to my baby would not be influenced after my decision
- I can drop out project at any moment.
- Under these conditions, I agree to participate

	Date	Signature
Investigator's name		
Mother or father's name		

9.4. ARISEN PUBLICATION FROM THE STUDY

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Randomized control trials

Safety and efficacy of inulin and oligofructose supplementation in infant formula: Results from a randomized clinical trial☆

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SUMMARY

Background & aims: The sterile newborn digestive tract is rapidly colonized after birth and feeding type could influence this process. Infant formulas try to mimic the bifidogenic effect of human milk using prebiotic supplementation. The aim of this study was to demonstrate the efficacy, safety and tolerance of a 0.8 g/dL Orafti®Synergy1 (oligofructose-enriched inulin) supplemented infant formula during the first 4 months of life.

Methods: In a double-blind, randomized, placebo-controlled and parallel trial, formula fed healthy term newborns were randomized to receive a control (controls) or SYN1 supplemented infant formula (SYN1). Breastfed newborns (BF) were also followed for comparison. Anthropometry, water balance, blood parameters, adverse events, stool frequency and characteristics and faecal microbiota were assessed.

Results: A total of 252 formula fed infants were randomized at birth (n 124 controls, n 128 SYN1) and 131 BF infants were recruited; after 4 months 68 controls, 63 SYN1 and 57 BF completed the study. SYN1 infants showed a microbiota composition closer to that of BF infants, with a trend towards higher *Bifidobacterium* cell counts, softer stools and a higher deposition frequency compared to controls. There were no differences between formulas in anthropometry and relevant adverse events, water balance or blood parameters.

Conclusion: A 0.8 g/dL SYN1-supplemented infant formula during the first 4 months of life is safe and effective, promoting a gut microbiota closer to that of breastfeeding.

This clinical trial was registered at Clinicaltrials.gov as Study on Fermentable Carbohydrates in Healthy Infants (number NCT00808756).

1. Introduction

Prenatally, the gastrointestinal tract is sterile. After birth, massive and rapid colonization of the digestive tract by microorganisms occurs as a part of the adaptation to extrauterine life. This colonization process is influenced by factors such as type of delivery, hygienic measures, prematurity, antibiotic therapy and feeding type.[1] Numerous studies have shown that the intestinal flora of infants fed with human milk includes higher proportions of *Bifidobacterium* and *Lactobacillus* than those of formula fed infants, who have a more complex flora with higher proportions of *Bacteroides*, *Enterobacteriaceae* and *Clostridium*.[2,3]

In recent decades, infant formulas have been improved with prebiotic supplementation, such as inulin and oligofructose, to mimic the bifidogenic effect of breastfeeding. Orafti®Synergy1 (SYN1) is a commercial combination of oligofructose and long-chain inulin (50:50).[4,5]

The beneficial effects of inulin and/or oligofructose on the intestinal microbiota have been demonstrated in adults.[6] Many studies that examined the use of formulas supplemented with long-chain fructan polysaccharides (i.e., long-chain inulin and fructooligosaccharides (FOS)) and galactooligosaccharide mixtures (GOS) in healthy newborns have shown an improvement in the gut flora

Abbreviations: BF, breastfeed; FOS, fructooligosaccharides; GOS, galactooligosaccharides; SYN1, Orafti®Synergy1.

☆ Conference presentation: 61st Anual Congres from the Asociación Espanyola de Pediatria (AEP). Granada 2012.

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composition,[7,8] frequency of depositions and a softer stool consistency.[8,9] In a recently published, randomized, controlled 4-week trial in newborns, a formula supplemented with 0.8 g/dL SYN1 had similar efficacy to a GOS:FOS (long-chain inulin) 90:10-supplemented formula. Both formulas promoted stool consistency and microflora composition closer to those of breastfed infants as compared to a control formula. In a consistency score ranging from 1 (watery stool) to 4 (hard stool), the breastfed group had a consistency slightly above 1, the two supplemented groups about 1.5 and the control group about 2.5. The mean number of bifidobacteria was around 12% higher among the breastfed infants and the supplemented formulas as compared to the control group.[10] To find out the effective dose of SYN1-supplemented formulas, the same study compared the 0.8 g/dL with a 0.4 g/dL SYN1-supplemented formula; they found that the 0.8 g/dL supplemented formula leaded to a higher number of depositions and to softer stools consistency. In addition, they observed a significant increase in *Bifidobaterium* among 0.8 g/dL SYN-supplemented infants as compared to the control group, while the 0.4 g/dL SYN-supplemented did not.[10]

There is a concern that a possible harmful effect by adding prebiotics in infant formulas may be produced by the induction to more watery stools, which could increase the risk of dehydration in some infants, as pointed out by the European Society for Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN).[11,12] The scientific committee on food of the European Commission reported that there is no conclusive data about water balance in infants fed with a prebiotic-supplemented formula.[12]

The aim of this study was to demonstrate the efficacy to modify gut microflora, tolerance and safety of a 0.8 g/dL SYN1-supplemented infant formula during the first 4 months of life.

2. Methods

2.1. Study design

The study was a double-blind, randomized, placebo-controlled and parallel trial. Two groups ($n = 100$ each) of neonates were randomized to infant formula either with or without 0.8 g/dL SYN1-supplemented formula until they were 4 months old (±5 days). A reference group of breastfed infants (BF; $n = 100$) was used for comparison (as recommended by the Committee on Nutrition of ESPGHAN).[13]

2.2. Study product descriptions

The test products were produced by BENEO-Orafti and had a general composition that complied with current EU standards (Commission Directive 2006/141/EC). Both infant formulas contained the same amounts of lactose, protein, fat and micronutrients (On-line Supplementary Table 1). The test substance was SYN1 (0.8 g/dl), a chicory-derived fructan formulation with an active fructan that is composed of approximately 50% oligofructose (degree of polymerization [DP] <10) and 50% long-chain inulin (DP ≥ 10). The control formula was supplemented with an amount of maltodextrin (which is not expected to have any effect on the gut) equivalent to the weight of the SYN1 in the SYN1-supplemented formula.

2.3. Blinding, randomization and allocation concealment

Four different infant formula containers were created (identified only by different colours), two for each study formula (to better protect the blinding of the intervention). The infant formulas were packed in sealed boxes labelled in such a way that the content of SYN1 and maltodextrin were unknown to both the study investigators and the parents of the study subjects. The code (correspondence between colour and treatment) was blinded to all of the investigators, participants and caregivers during all the study progress (until the end of the study after all data were introduced in the data base and statistically processed) and was not blinded to manufacturer.

The random list of treatments was prepared by an investigator (not involved in recruiting families and assigning treatments) using the computer program EPIDAT (http://www.paho.org/spanish/sha/epidat.htm) (Servizo de Epidemioloxía da Dirección Xeral de Innovación e Xestión da Saúde Pública da Consellería de Sanidade (Xunta de Galicia), A Coruña, Spain). Allocation concealment was ensured by sequentially numbered sealed envelopes not accessible to investigators until the informed consent was signed.

2.4. Study population

All infants were recruited from Hospital Universitari de Tarragona Joan XXIII and Hospital Universitari Sant Joan de Reus (in Spain), mainly during their hospital stay for the birth or during the four following weeks (by phone call).

The inclusion criteria of infants were to be healthy, born at term (≥37 weeks gestational age), with normal birth weight (between the 3rd and 97th percentiles for gestational age) according to current Spanish references,[14] having at recruitment an age below 4 weeks and normal feeding behaviour or skills. Feeding type of formula milk or human milk had to be more than 90% of energy intake at recruitment to be included in the study as formula fed infant or breastfed infant respectively. There was no control of the infant formula or human milk consumed prior to enrolment. No complications in breastfeeding before hospital discharge and mother's firm conviction to exclusively breastfed her infant during at least the first 4 months of life were inclusion criteria for breastfed

Table 1
Baseline characteristics of infants.

	Control[a]	SYN1[b]	Breastfed
Gender (*n* male/*n* female)	67/57	62/66	66/70
Delivery Type (*n* vaginal/*n* caesarean)	84/40	104/24*	104/32
Gestational age (Mean (SD))	39.9 (1.3)	39.7 (1.1)	39.8 (1.3)
Birth weight (g) (Mean (SD))	3235 (346)	3289 (385)	3302 (346)
Birth length (cm) (Mean (SD))	49.5 (2.0)	49.7 (1.8)	49.8 (1.6)
Birth head circumference (cm) (Mean (SD))	34.2 (1.3)	34.3 (1.2)	34.5 (1.2)
Maternal education level (*n*(%))			
Preliminary education level	1 (0.9)	0 (0)	0 (0)
Primary education level	11 (9.3)	9 (7.9)	4 (3.3)
Lower secondary education level	35 (29.7)	43 (37.7)	16 (13.2)
Upper secondary education level	36 (30.5)	25 (21.9)	20 (16.5)
Post-secondary education level	12 (10.2)	13 (11.4)	23 (19.0)
First stage of tertiary education level	23 (19.5)	24 (21.1)	56 (46.3)
Second stage of tertiary education level	0 (0)	0 (0)	2 (1.7)

*$p < 0.05$ SYN1 group vs. control group.
[a] Infants fed with the control formula.
[b] Infants fed with SYN1-supplemented formula.

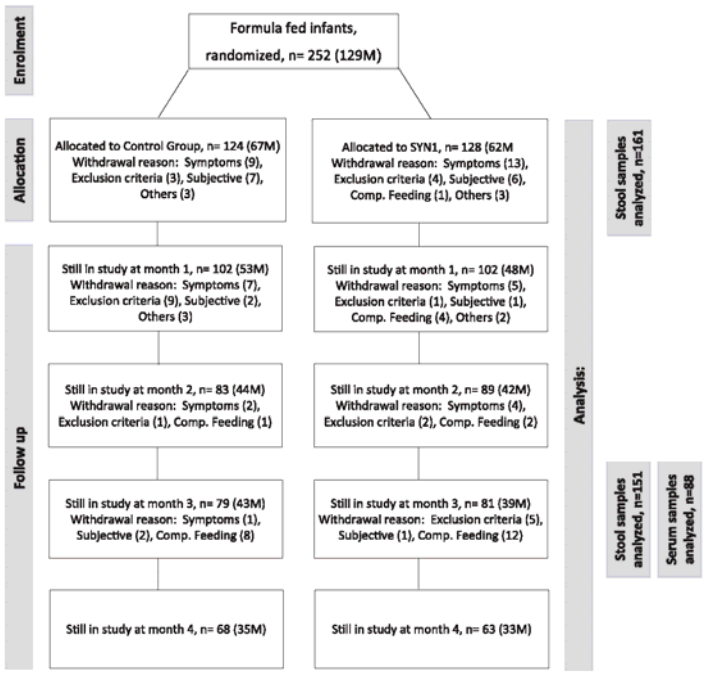

Fig. 1. Recruitment, randomization, follow-up and withdrawals at each study time point. M: males. Symptoms includes all infants who abandoned the study to switch to another infant formula due to digestive symptoms (see Methods); *subjective* includes the parents' subjective perceptions of formula acceptance; *exclusion criteria* includes all infants receiving medication; *comp. feeding* refers to the introduction of complementary foods to the infants' diet; *others* includes contact losses and unknown reasons.

infants as well. The exclusion criteria for recruitment were more than 4 weeks of life; antibiotic treatment; serious respiratory, neurological, gastrointestinal or metabolic disorders; and infections or other serious diseases that could hinder growth. During the study, the exclusion criteria were antibiotic treatment and switching to other formulas or complementary feeding for more than 10% of the total energy intake or for more than 3 days.

Before the research team approached the families, the investigators checked the chosen feeding type, to inform them properly. Breastfeeding was encouraged and supported during all the study period. No information about infant formula distribution (to infants in formula fed groups) was given to families of breastfeed infants to avoid any negative influence on breastfeeding decision. Breastfed infants who were included in the study from birth but switched to exclusive formula feeding during the first 4 weeks of life, were then informed and had the possibility to be randomized and allocated in 1 of the 2 study formula groups.

2.5. Outcome measures

The primary outcome to assess the efficacy of the SYN1-supplemented infant formula was the gut microflora. Secondary outcomes to assess efficacy were stools frequency, stools consistency and infections incidence. Main outcome measures to assess safety were growth, water balance and biochemical parameters. Digestive symptoms, crying behaviour, dietary intake, maternal report of infant's acceptance of study formula were analysed to assess tolerance.

2.5.1. Microbiota analyses

The spot stool samples were collected at inclusion and at 3 months (±5 days). The microflora analyses were conducted only for the infants whose initial (inclusion) sample collection was performed during the first week of life. The samples were preserved at −80 °C at the study centers until they were shipped to a central laboratory (Institut für Mikroökologie, Herborn, Germany) for analysis (<6 months). The bacterial flora analyses of the stool samples (in log cfu/g faeces) for *Bifidobacterium*, *Bacteroides*, *Enterobacteriaceae*, *Clostridium coccoides*, *Clostridium leptum* (clostridial cluster IV) and total bacteria were conducted for a subgroup using SYBR Green quantitative PCR (qPCR) with an ABI 7300 Real-Time PCR System (Applied Biosystems, Darmstadt, Germany). The DNA was extracted from the stool samples using an Easy Mag DNA isolation system (BioMerieux, Nuertingen, Germany) according to the manufacturer's instructions. The detailed method and primers used have been described elsewhere.[15,17] In some groups, some

Table 2
Results of microbiota analysis in faecal samples (cross sectional comparison of infants SYN1-supplemented vs. control).

	Control[a]		SYN1[b]		Breastfed[c]	
	$n <$ LoD	Median (IQR)	$n <$ LoD	Median (IQR)	$n <$ LoD	Median (IQR)
AT RECRUITMENT	$n = 55$		$n = 53$		$n = 53$	
Bacteroides (log cfu/g faeces)	12	6.88 (5.22, 9.31)	21	6.01 (5.00, 7.80)	14	7.07 (5.00, 10.16)
Bifidobacterium (log cfu/g faeces)	14	6.29 (5.00, 8.86)	13	6.30 (5.04, 9.27)	12	6.00 (5.03, 7.59)
Enterobacteriaceae (log cfu/g faeces)	27	5.11 (5.00, 9.99)	26	5.10 (5.00, 9.47)	26	5.38 (5.00, 9.37)
Total counts (log cfu/g faeces)	0	10.60 (10.12, 11.03)	0	10.26 (9.57, 10.72)*	0	10.42 (9.07, 11.08)
MONTH 3	$n = 50$		$n = 52$		$n = 49$	
Bacteroides (log cfu/g faeces)	10	9.14 (5.70, 10.25)	15	7.45 (5.00, 9.10)*	14	7.68 (5.00, 10.32)
Bifidobacterium (log cfu/g faeces)	3	8.64 (6.91, 10.01)	2	9.59 (8.55, 10.17)	1	9.62 (9.16, 10.23)
Enterobacteriaceae (log cfu/g faeces)	4	9.28 (8.50, 9.88)	5	8.71 (7.88, 9.33)*	15	8.51 (5.00, 9.35)
T. counts (log cfu/g faeces)	0	11.13 (10.37, 11.46)	0	10.87 (10.44, 11.32)	0	10.67 (9.79, 11.32)

n: Total number analysed, $N <$ LoD: Number of faecal samples below the detection limit; *$p < 0.05$ SYN1 group vs. control group.
[a] Infants fed with the control formula.
[b] Infants fed with SYN1-supplemented formula.
[c] Breastfed.

stool samples had bacterial counts below the detection limit (<5 log cfu/g faeces). In these cases, the detection limit was imputed in the statistical analysis.

2.5.2. Stool frequency and characteristics

The parents recorded the number of depositions and their characteristics in a 2-day diary before each study visit (at 1, 2, 3 and 4 months). The characteristics for each deposition were recorded using the Bristol scale (categories from 1 to 7, hardest to softest respectively).[18] The consistency score was calculated as = Σ (type of stool × number of depositions of each type), all divided by the total number of depositions.

2.5.3. Medical history

Any illness events that infants underwent were recorded at all visits.

2.5.4. Anthropometry

The infants' birth weights, lengths and head circumferences were obtained from their hospital records. The nude weight and length of each infant were determined in duplicate with a SECA 336 baby scale (precision: ±10 g) and a SECA 232 stadiometer (precision: ±1 mm), respectively. The skinfold thickness (tricipital and subscapular) were measured in triplicate using a Holtain caliper (precision: ±0.2 mm). Head and mid-upper arm circumferences were measured in duplicate using a SECA non-extendable insertion tape (precision: ±1 mm).

2.5.5. Water balance and blood biochemical parameters

The urinary analyses were performed at study months 1 and 3. Urinary Cl (mEq L^{-1}), K (mEq L^{-1}) and Na (mEq L^{-1}) concentrations were determined using indirect potentiometry. The Na/K ratio was calculated to assess the level of hydration. The creatinine (mEq dL^{-1}) concentrations were determined using the Jaffe reaction method with a Siemens ADVIA 2400 (Siemens Medical Solutions and Diagnostics, Dublin, Ireland) in accordance with standardized protocols. The osmolarity (mOsm L^{-1}) was measured using the USC method with an ARKAY Osmo Station OM-6050 (A. Menarini Diagnostics, Valkenswaard, The Netherlands).

A blood sample was drawn from a vein access at 3 months of life in a subgroup of infants (aiming an $n = 30$ per group). Mothers were invited to have their infants participating in the blood collection until the desired number in the respective group was achieved.

Briefly the Cl, K and Na concentrations were determined using indirect potentiometry; total proteins were determined using the Biuret method; albumin was measured using the bromocresol purple method; and urea and cholesterol were measured using an enzymatic method with a Siemens ADVIA 2400 (Siemens Medical Solutions and Diagnostics, Dublin, Ireland) in accordance with standardized protocols. Prealbumin levels were analysed via nephelometry using a Siemens BN II (Siemens Medical Solutions and Diagnostics, Dublin, Ireland).

2.5.6. Formula intake and acceptance

A two days food diary was completed by families two days prior to study visits. Outcome measures were formula intake (ml/day·kg) and energy intake (kcal/day/kg). Mothers reported infant's acceptance of study formula monthly, answering one of five possible categories (very good, good, moderate, poor and very poor).

2.5.7. Adverse events

Vomiting, regurgitation, digestive symptoms (e.g., discomfort, belly pain or cramps) and amount of time spent crying were assessed based on 2-day diaries or participants' recollections during clinic visits. Other relevant adverse events (regardless of whether they were related to the study formula) that occurred during the follow-up period and the mothers' perceptions of the infants' tolerance of the formula (scored from very good to very poor on a Likert scale) were collected via telephone calls.

2.6. Statistical analyses

The sample size (64 per group) was calculated to detect a difference of 0.5 SD in weight gain (as the main safety outcome measure) over the study period. During the field work, we envisaged a dropout rate higher than the forecasted (35%). Therefore in

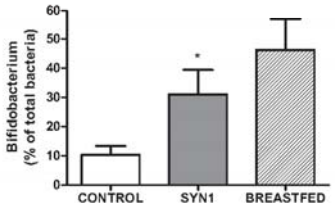

Fig. 2. Percentages of *Bifidobacterium* in the stool samples of the feeding groups at infant's age 3 months. The white bars indicate the control formula group, the grey bars indicate the prebiotic-supplemented formula group and the filled bars indicate the breastfed group. *$p < 0.05$ vs. control group.

Table 3
Stool frequency and consistency by feeding groups (mean of 2-days diary).

	Control[a]	SYN1[b]	Breastfed
	Median (IQR)	Median (IQR)	Median (IQR)
MONTH 1			
Stool frequency (n/day)	2.5 (2.0, 4.0)	4.0 (2.5, 5.1)***	5.0 (2.5, 7.0)
Stool consistency score (1–7)	6.0 (4.7, 6.0)	6.0 (6.0, 6.0)***	6.3 (6.0, 7.0)
MONTH 2			
Stool frequency (n/day)	2.0 (1.5, 3.0)	2.5 (2.0, 3.5)**	3.0 (1.5, 5.0)
Stool consistency score (1–7)	6.0 (4.3, 6.0)	6.0 (6.0, 6.0)****	6.8 (6.0, 7.0)
MONTH 3			
Stool frequency (n/day)	2.0 (1.5, 2.5)	2.5 (1.5, 3.0)**	2.0 (1.0, 4.0)
Stool consistency score (1–7)	6.0 (4.0, 6.0)	6.0 (6.0, 6.0)***	6.5 (6.0, 7.0)
MONTH 4			
Stool frequency (n/day)	2.0 (1.5, 2.5)	2.5 (1.5, 3.0)*	1.5 (1.0, 3.0)
Stool consistency score (1–7)	6.0 (4.0, 6.0)	6.0 (6.0, 6.0)***	6.5 (6.0, 7.0)

*$p < 0.05$ SYN1 group vs. control group.
**$p < 0.01$ SYN1 group vs. control group.
***$p < 0.001$ SYN1 group vs. control group.
****$p = 0.001$ SYN1 group vs. control group.
[a] Infants fed with the control formula.
[b] Infants fed with SYN1-supplemented formula.

order to not lose statistical power we randomized 52 additional participants after the first 200 formula fed infants had been recruited. Only the formula fed (randomized) groups were included in the per protocol analyses. The descriptive results were expressed as means (±SD) or medians and interquartile ranges after assessing the normal distribution of variables with a Kolmogorov–Smirnov test. The faecal microbiota concentrations were converted to base-10 logarithms. *T*-tests or Mann–Whitney *U*-tests were used for statistical comparisons during the cross sectional analysis between feeding groups, as requested by normally distributed or none normally distributed variables. Pearson's chi-squared test was used for the statistical comparison of the categorical data. In addition, we performed intention-to-treat analyses to test for differences between feeding groups as well. A linear regression analysis including formula type and bacterial counts was performed to show the effects on faecal consistency and frequency. The statistical significance was accepted at $p < 0.05$. The data management and statistical analyses were conducted using the SPSS Statistics software version 17.0 (Chicago, IL).

2.7. Ethical considerations

The study was performed according to the Declaration of Helsinki II Principles and was approved by the local ethics committees. All parents or caregivers of the participating infants provided written informed consent to the study in accordance with the local ethical committees' requirements. The study followed the recommendations of the CONSORT guidelines.[19] This clinical trial was registered at Clinicaltrials.gov as Study on Fermentable Carbohydrates in Healthy Infants (number NCT00808756).

3. Results

3.1. Study population

Three hundred and eighty-eight infants were recruited between September 2008 and June 2010 (BF; $n = 136$). At recruitment all formula fed infants were not having any human milk, and all breastfed infants were not having any formula milk. Five breastfed infants switched to exclusively formula feeding during the first four weeks of life, and accepted to be included in the randomized clinical trial formula fed groups (1 control group and 4 SYN1 group). The formula groups (control and SYN1) were comparable in terms of all of the anthropometrical variables, gestational age at birth, maternal education level and gender distribution (Table 1). The control group had a higher proportion of caesarean sections ($p < 0.05$) than the SYN1-suplemented formula group. However, no differences in delivery type were found in the subgroup from which faecal samples were analysed.

One hundred and eighty-eight infants completed the entire fourth months study protocol (68 in the control group, 63 in the SYN1 group, and 57 in the breastfed group; Fig. 1). There were not significant differences between formula groups in the day of life that they begun the intervention (median day 3 (IQR: 2, 5) for infants in control group and 3 (IQR: 2, 7) for infants in SYN1 group) we neither found differences on distribution of infants recruited after hospital discharge. There were no differences between the formula groups in the total numbers of withdrawals or in their reasons for withdrawing from the study.

3.2. Efficacy

Microbiota in infants' faecal samples

The faecal samples were collected at recruitment, before started with study milk formula ($n = 161$) and at month 3 ($n = 151$). Table 2 shows the results of the bacteria analyses of the faecal samples. The numbers of analysed faecal samples below the detection limit (5 log cfu/g faeces) were comparable between the formula groups for all of the bacterial strains. However, because more than 50% of the samples showed results below the detection limit for *C. coccoides* and *C. leptum*, these bacteria were omitted (Table 2). At baseline all bacteria cell counts were similar in both formula groups, apart from slightly lower total bacteria counts in the SYN1. At month 3, there was a trend toward higher *Bifidobacterium* cell counts in the SYN1 group compared to the control group ($p = 0.061$) (Table 2). When we analysed the proportion of bifidobacteria (%) in the total bacteria, this differences became significant ($p < 0.05$) (Fig. 2). The *Bifidobacterium* counts in the SYN1 group were more similar to those of the breastfed group than to those of the control group (as cell counts and as proportion, Table 2 and Fig. 2). The numbers of *Bacteroides* and *Enterobacteriaceae* were significantly lower in the SYN1 group than in the control group and were more similar to those of the breastfed infants (Table 2).

3.2.2. Stool frequency and characteristics

The SYN1 group had a significantly higher frequency of depositions than did the infants fed with the control formula (Table 3) at all study time points. The stools were also softer in the SYN1 group than in the control group and were more like those of the breastfed infants (Table 3). Of note, the stool consistency for the SYN1 group infants was more constant that those observed in the control group.

Linear regression analyses with two covariates (i.e., formula and bacterial strain) were performed and revealed a significant direct association of the SYN1-supplemented formula ($p < 0.05$) and the level of gut *Bifidobacterium* ($p < 0.01$) with the faecal consistency score, explaining up to 13.5% of its variability.

In a second linear regression model we found a direct association of the SYN1 formula ($p < 0.05$) and an inverse association of *Enterobacteriaceae* counts in the gut ($p < 0.001$) with the stools frequency. The variability of the stool frequency was explained up to 18.2% by both the study formula and *Enterobacteriaceae* levels.

Table 4
Anthropometric characteristics of infants by feeding group at all time points.

	Control[a] mean (SD)		SYN1[b] mean (SD)		Breastfed mean (SD)	
	PP	ITT	PP	ITT	PP	ITT
MONTH 1	(*n* = 102)	(*n* = 106)	(*n* = 102)	(*n* = 109)	(*n* = 87)	(*n* = 99)
Weight (g)	4185 (433)	4175 (430)	4169 (446)	4167 (440)	4318 (484)	4299 (496)
Length (cm)	53.8 (1.9)	53.8 (1.9)	54.1 (2.0)	54[2]	54.4 (1.6)	54.3 (1.7)
Head circumference (cm)	37.3 (1.1)	37.3 (1.1)	37.2 (1.3)	37.2 (1.2)	37.5 (1.1)	37.5 (1.1)
Tricipital skinfold (mm)	6.4 (1.0)	6.4 (0.9)	6.2 (0.9)	6.2 (0.9)	6.6 (1.0)	6.6[1]
Subscapular Skinfold (mm)	6.6 (1.0)	6.5 (1)	6.4 (1.1)	6.4 (1.1)	6.7 (1.2)	6.7 (1.2)
MONTH 2	(*n* = 83)	(*n* = 96)	(*n* = 89)	(*n* = 103)	(*n* = 79)	(*n* = 94)
Weight (g)	5165 (523)	5158 (526)	5162 (539)	5159 (529)	5217 (611)	5219 (593)
Length (cm)	57.4 (1.9)	57.4 (1.9)	57.6 (2.0)	57.6[2]	57.8 (1.7)	57.7 (1.7)
Head circumference (cm)	39.0 (1.1)	39 (1.1)	39.0 (1.3)	38.9 (1.2)	39.2 (1.1)	39.2 (1.1)
Tricipital skinfold (mm)	7.3 (1.2)	7.3 (1.2)	7.2 (1.0)	7.2[3]	7.3 (1.0)	7.4 (1.1)
Subscapular Skinfold (mm)	7.1 (1.2)	7.1 (1.2)	7.1 (1.3)	7.1 (1.3)	7.2 (1.1)	7.2 (1.1)
MONTH 3	(*n* = 79)	(*n* = 94)	(*n* = 81)	(*n* = 101)	(*n* = 68)	(*n* = 90)
Weight (g)	6008 (580)	6008 (619)	6003 (637)	5978 (637)	5902 (732)	5899 (699)
Length (cm)	60.7 (1.9)	60.6 (1.9)	60.8 (2.1)	60.7 (2.1)	60.7 (1.8)	60.7 (1.8)
Head circumference (cm)	40.4 (1.2)	40.4 (1.1)	40.4 (1.3)	40.3 (1.3)	40.5 (1.2)	40.5 (1.8)
Tricipital skinfold (mm)	8.1 (1.4)	8.1 (1.4)	7.9 (1.3)	7.9 (1.3)	7.9 (1.3)	7.9 (1.3)
Subscapular Skinfold (mm)	7.2 (1.1)	7.2 (1.1)	7.2 (1.3)	7.2 (1.2)	7.3 (1.3)	7.2 (1.3)
MONTH 4	(*n* = 68)	(*n* = 92)	(*n* = 63)	(*n* = 103)	(*n* = 57)	(*n* = 89)
Weight (g)	6741 (619)	6770 (716)	6746 (728)	6698 (726)	6491 (797)	6513 (753)
Length (cm)	63.6 (1.8)	63.6 (1.9)	63.5 (2.4)	63.3 (2.4)	63.2 (2.0)	63.3 (2.1)
Head circumference (cm)	41.6 (1.2)	41.6 (1.2)	41.7 (1.4)	41.4 (1.3)	41.6 (1.3)	41.5 (1.2)
Tricipital skinfold (mm)	8.5 (1.5)	8.6 (1.5)	8.3 (1.5)	8.3 (1.4)	8.5 (1.7)	8.5 (1.7)
Subscapular Skinfold (mm)	7.4 (1.2)	7.4 (1.3)	7.2 (1.3)	7.3 (1.3)	7.1 (1.4)	7.1 (1.3)

PP: per protocol analyses, ITT: Intention-to-treat analyses. *T*-test between formula fed groups not significant for any anthropometrical variables.
[a] Infants fed with the control formula.
[b] Infants fed with SYN1-supplemented formula.

3.2.3. Medical history

We did not find any significant difference in the mean number of infections between formula groups (data not shown).

3.3. Safety and tolerance

3.3.1. Anthropometry

There were no significant differences between the formula groups in any of the anthropometrical parameters (i.e., weight, length, head, waist and arm circumference or tricipital and subscapular skinfold results) at any of the time points (Table 4).

3.3.2. Water balance and blood biochemical parameters

At month 1, the Na, Na/K, Cl, and creatinine concentrations and the osmolarity were similar in the urine samples of the formula groups (Table 5). In the SYN1 supplemented group, the K concentration was lower and closer to that of the BF group (Table 5). At month 3, there were no differences in any of the water balance parameters between the formula groups (Table 5).

Blood samples were taken from 88 infants at 3 months. The results were comparable between the formula groups for Na, Cl or K ions, along with those for proteins, albumin, prealbumin and cholesterol (Table 5). The urea concentration was slightly lower in the SYN1 group and closer to that of the BF infants, compared with the control group.

3.3.3. Formula intake and acceptance

The average total formula intake (ml day $^{-1}$ kg $^{-1}$) or energy intake (kcal day $^{-1}$ kg $^{-1}$) (Table 6) was comparable between the formula groups throughout the follow-up period. The mothers' perceptions of the infants' formula acceptance were similar in both formula groups at all time points (Table 6).

3.3.4. Possible adverse events

There were no differences between groups in terms of regurgitation and frequency of digestive discomfort throughout the study (*n*/day) (Table 6). The amount of time spent crying (min/day) of the SYN1 group was slightly longer compared to that of the control group at 4 months but not at the other time points (Table 6).

A total of 67 possible adverse events were reported (e.g., frequent vomiting, regurgitation or digestive discomfort; loose stools; skin rash; bloody stools; and lack of weight gain). These events occurred at equal rates in both groups, with the exception of slightly fewer instances of loose stools in the SYN1 group compared to the control group (2% vs. 8%; $p < 0.05$).

3.3.5. Intention-to-treat analyses

Finally, we performed intention-to-treat analyses to test for differences between feeding groups as well. With the intention-to-treat analyses we obtained all the same final results for growth, water balance, biochemical blood parameters and digestive symptoms. Faecal samples of withdrawn participants were not analysed.

4. Discussion

This is the first randomized clinical trial to comprehensively demonstrate the safety and efficacy of a 0.8-g/dl SYN-1-supplemented infant formula for healthy, term infants during the first 4 months of life. Although a previous study assessed the effects of SYN1 on newborns,[10] the study period was only four weeks, and the water balance was not assessed. Our study extended the observation time to assess whether the softer and more frequent stools that were observed affected the infants' growth or hydration statuses.

4.1. Efficacy

The efficacy of the SYN1-supplemented formula was demonstrated by both the microflora analyses and the improved stool patterns.

Table 5
Description of laboratory parameters by feeding group.

	Control[a]	SYN1[b]	Breastfed
	Median (IQR)	Median (IQR)	Median (IQR)
Urinary parameters			
MONTH 1	(*n* = 70)	(*n* = 67)	(*n* = 65)
Urinary Cl (mEq/L)	19.0 (11.0, 30.3)	17.0 (10.0, 26.0)	12.0 (7.0, 19.0)
Urinary K (mEq/L)	20.5 (12.7, 29.1)	15.5 (9.9, 23.2)*	12.1 (8.8, 17.3)
Urinary Na (mEq/L)	17.0 (9.8, 26.3)	12.0 (7.0, 23.0)	5.0 (3.0, 9.0)
Urinary Na/K ratio	0.85 (0.6, 1.3)	0.9 (0.5, 1.2)	0.4 (0.2, 0.8)
Urinary Creatinine (mEq/dL)	8.9 (5.9, 14.4)	8.4 (4.7, 12.0)	5.9 (4.0, 8.8)
Urinary Osmolarity (mosm/L)	150.5 (99.3, 210.3)	115.0 (80.0, 192.0)	90.0 (54.5, 145.0)
MONTH 3	(*n* = 59)	(*n* = 60)	(*n* = 49)
Urinary Cl (mEq/L)	22.0 (9.0, 47.0)	19.5 (10.3, 31.8)	14.0 (9.0, 23.0)
Urinary K (mEq/L)	19.8 (12.3, 33.1)	17.8 (10.7, 27.9)	13.6 (8.7, 22.1)
Urinary Na (mEq/L)	22.0 (7.0, 50.0)	17.0 (7.3, 40.8)	5.0 (2.5, 10.5)
Urinary Na/K ratio	0.8 (0.4, 1.7)	1.1 (0.6, 1.5)	0.4 (0.2, 0.7)
Urinary Creatinine (mEq/dL)	11.8 (6.5, 18.9)	9.4 (5.9, 17.5)	9.0 (5.8, 13.3)
Urinary Osmolarity (mosm/L)	159.0 (96.8, 267.8)	143.0 (73.8, 253.3)	104.0 (50.0, 172.0)
	Mean (SD)	Mean (SD)	Mean (SD)
Blood parameters			
MONTH 3	(*n* = 29)	(*n* = 37)	(*n* = 22)
Cholesterol (mg/dL)	130.2 (15.9)	132.7 (20.4)	157.8 (27.2)
Urea (mg/dL)	22.6 (3.3)	19.6 (3.6)**	13.8 (3.7)
Proteins (g/dL)	6.0 (0.4)	6.0 (0.4)	5.9 (0.4)
Albumin (g/dL)	4.3 (0.4)	4.2 (0.4)	4.2 (0.2)
Prealbumin (mg/dL)	20.1 (6.9)	21.0 (9.2)	16.3 (5.1)
Serum Na (mEq/L)	138.6 (1.5)	138.6 (1.8)	137.8 (2.1)
Serum K (mEq/L)	5.5 (0.5)	5.4 (0.6)	5.2 (0.4)
Serum Cl (mEq/L)	105.4 (1.7)	106.1 (2.0)	106.4 (2.1)
Serum pH	7.3 (0.04)	7.3 (0.05)	7.4 (0.1)
Serum base excess (mmol/L)	−1.4 (1.8)	−1.6 (2.1)	−0.5 (2.1)
Serum Calcium (mmol/L)	1.3 (0.1)	1.3 (0.1)	1.3 (0.1)

*$p < 0.05$ SYN1 vs. control group.
**$p < 0.01$ SYN1 vs. control group.
[a] Infants fed with the control formula.
[b] Infants fed with SYN1-supplemented formula.

The composition of the microflora differed between the two groups, having the inulin-oligofructose mixture a bifidogenic effect. The infants who were supplemented with 0.8 g/dL SYN1 showed a trend toward increased total counts of *Bifidobacterium* in the faecal flora when compared with the infants who were fed a non-supplemented formula. This is consistent with the finding of a significantly higher proportion of bifidobacteria in the SYN1 group compared with the control group (Fig. 2). Similar results were reported by Veereman-Wauters et al., who reported an increase in *Bifidobacterium* at days 14 and 28 of life compared with day 3 in infants fed with SYN1 0.8 g/dL and FOS (long-chain inulin):GOS formulas and found no differences in the SYN1 0.4 g/dL and control groups.[10] In our study, this positive effect persisted for at least 3 months. In contrast, other authors were unable to find significant differences in *Bifidobacterium* in infants fed prebiotic or standard formulas after 6 weeks of intervention.[9] In this study, lower concentrations of *Enterobacteriaceae* and *Bacteroides* were identified in the SYN1 group compared with the control group. These changes in the SYN1-supplemented formula infants were similar to those observed between the formula fed and BF infants.[3,20] In addition, the SYN1-supplemented infants had bacteria counts closer to those of the breastfed infants.

The stool frequency and consistency pattern of the SYN1-supplemented infants were associated with a higher number of depositions and a softer consistency throughout the study period when compared with the nonsupplemented infants (Table 3). This pattern was more similar to the pattern in breastfed infants. The softer and more frequent stools observed in the SYN1-supplemented group may partially be explained by the higher *Bifidobacterium* colonization noted previously, as shown by the linear regression analyses.

This improvement of stool frequency and consistency is of great interest, given that this may partly reduce the adverse effects (i.e., constipation) of feeding infants a standard formula rather than breastfeeding.[21] Tunc VT et al. showed a hard stool deposition in 1.1% of breastfed infants and in 9.2% of formula fed infants ($p = 0.001$).[22] Although other authors have reported similar results, none of them have assessed the possible side effects of having softer and more frequent stools.[8,10] It is worth stressing that the increases in the number of depositions and in stool softness were not accompanied by changes in the hydration status, as demonstrated by the water balance analyses noted previously.

Van Stuijvenberg M et al.[23] showed that there was no difference in the number of fever episodes between the prebiotic formula (FOS/GOS) and control formula, neither during the intervention (6 months) nor later on (12 months). Our results are consistent with those from Van Stuijvenberg M et al. and do not contradict those from Arslanoglu et al.[24] In this randomized clinical trial, Arslanoglu et al. did not find any difference during the first 4 months of life between infants fed supplemented or not with a FOS/GOS infant formula. However, they found lower infection incidence from 4th to 6th month of life. Possibly, we think that a longer-term follow-up of the infants in our study would help to elucidate the possibility of preventing infections by prebiotic supplementation in infants.

4.2. Safety and tolerance

The prebiotics' fermentation processes in the intestine may be associated with the onset of gastrointestinal symptoms (e.g., vomiting, regurgitation, diarrhoea or flatulence) and irritability (e.g., agitation and crying). The presence of these symptoms can lead to the discontinuation of prebiotic intake or could interfere with the child's growth. Consequently, we assessed whether the formula with prebiotics influenced the overall intake of milk and calories. We noted similar formula intakes and acceptance in the two groups of infants. Thus, the study results suggest that SYN-1 supplementation is safe in terms of infant ingestion. Some authors have reported a similar intake for the standard and prebiotic-supplemented formulas in infants,[8,10] whereas others have not assessed this parameter.[7]

A major concern was evaluating the infants' growth during the first quarter. All of the infants exhibited similar growth patterns. The infants fed with the prebiotic formula experienced similar weight, length and head circumference gains during the first four months of life, and the fatty body composition assed by skinfolds was similar for both formula groups. Although two different meta-analyses have shown that prebiotic supplementation is associated with slightly greater weight gains,[25,26] some previous studies have reported no significant differences in growth in preterm and term infants fed with prebiotic-supplemented formulas.[8,10,27] Moreover, a Cochrane Review concluded that the prebiotic supplementation

Table 6
Formula intake and acceptance, crying time and digestive symptoms of infants by feeding group at all time points.

	Control[a]	SYN1[b]	Breastfed
MONTH 1	(*n* = 102)	(*n* = 102)	(*n* = 87)
Formula intake (ml/Kg·day) (Mean (SD))	184.5 (29.7)	188.3 (34.2)	–
Energy intake (Kcal/Kg·day) (Mean (SD))	123.27 (19.82)	122.21 (22.22)	–
Formula acceptance (*n* (%))			
Very good	50 (78.1)	48 (75)	–
Good	14 (21.9)	15 (23.4)	–
Moderate	0 (0)	1 (1.6)	–
Regurgitation (*n*/day) (Median (IQR))	2 (0, 3.5)	1 (0, 2.6)	2.5 (0.5, 4.5)
Digestive discomfort (*n* (%))	39 (38.2)	27 (26.5)	34 (40.5)
Crying time (min/day) (Median (IQR))	41 (18, 81)	35.5 (20, 80)	30 (15, 60)
MONTH 2	(*n* = 83)	(*n* = 89)	(*n* = 79)
Formula intake (ml/Kg·day) (Mean (SD))	165.5 (23.3)	166.6 (24.5)	–
Energy intake (Kcal/Kg·day) (Mean (SD))	110.57 (15.58)	108.14 (15.93)	–
Formula acceptance (*n* (%))			
Very good	41 (66.1)	47 (72.3)	–
Good	20 (32.3)	18 (27.7)	–
Moderate	1 (1.6)	0 (0)	–
Regurgitation (*n*/day) (Median (IQR))	0.5 (0, 3.3)	0.5 (0, 2.5)	1.5 (0, 4)
Digestive discomfort (*n* (%))	19 (23.5)	15[17]	13 (16.5)
Crying time (min/day) (Median (IQR))	26 (9, 62)	25 (10, 43)	25 (10,50)
MONTH 3	(*n* = 79)	(*n* = 81)	(*n* = 68)
Formula intake (ml/Kg·day) (Mean (SD))	153.3 (30.9)	152.9 (24.0)	–
Energy intake (Kcal/Kg·day) (Mean (SD))	102.38 (20.68)	99.27 (15.55)	–
Formula acceptance (*n* (%))			
Very good	51 (85)	53 (86. 9)	–
Good	9 (15)	7 (11.5)	–
Moderate	0 (0)	1 (1.6)	–
Regurgitation (*n*/day) (Median (IQR))	0.5 (0, 2.5)	0.5 (0, 2.3)	1.5 (0, 2.5)
Digestive discomfort (*n* (%))	2 (2.6)	5 (6.3)	5 (7.4)
Crying time (min/day) (Median (IQR))	19 (6, 40)	20 (6, 35)	20.5 (10, 39)
MONTH 4	(*n* = 68)	(*n* = 63)	(*n* = 57)
Formula intake (ml/Kg·day) (Mean (SD))	140.8 (26.4)	144.2 (25.8)	–
Energy intake (Kcal/Kg·day) (Mean (SD))	94.04 (17.66)	93.60 (16.74)	–
Formula acceptance (*n* (%))			
Very good	46 (93.9)	39 (84.8)	–
Good	3 (6.1)	7 (15.2)	–
Moderate	0 (0)	0 (0)	–
Regurgitation (*n*/day) (Median (IQR))	0.5 (0, 2)	0 (0, 1.3)	0.8 (0, 2.8)
Digestive discomfort (*n* (%))	1 (1.5)	2 (3.2)	2 (3.5)
Crying time (min/day) (Median (IQR))	15 (3,26)	20 (10, 40)*	18 (10, 32)

*$p < 0.05$ SYN1 group vs. control group.
[a] Infants fed with the control formula.
[b] Infants fed with SYN1-supplemented formula.

of infant formula had no consistent effects on infant growth.[26] A recent study of infants who consumed prebiotics during the first year of life revealed no differences in growth during this long follow-up period.[28] In accordance, the most recent systematic review and commentary by the ESPGHAN Committee on Nutrition concluded that the supplementation of infant formula with prebiotics has no adverse effects on growth in healthy term infants.[29]

The infant formula had no effect on the subjects' reasons for dropping out of the study. The digestive symptoms (e.g., regurgitation or gastrointestinal symptoms) reported as reasons for withdrawal were those that are characteristic of this period of infancy (for both formula and breastfed infants). Typical gastrointestinal symptoms (e.g., regurgitation, vomiting and digestive discomfort) and crying behaviour during the follow-up period were comparable between the formula groups. Similar results have been reported by other authors.[8,10]

The SYN1 supplementation group had softer and more frequent stools, which could be regarded as an indicator of positive gut health and was not associated with an impaired water balance. Both the osmolarity of the urine and the sodium-potassium ratio were similar between formula fed groups, reflecting comparable hydration levels. Both formula groups also had similar urinary concentrations of ions and creatinine, with the exception of K at month 1, which was slightly lower in the SYN1-supplemented group and was more similar to that of the breastfed infants than that of the control group. These results have special relevance because, to the best of our knowledge, none of the previously published trials assessing the effects of prebiotic supplementation in infant formulas have measured this safety parameter.

In addition to the urinary determinations, we also measured the blood serum parameters to assess safety (Table 5). The plasmatic ion and bicarbonate concentrations and pH values were similar in both of the formula fed groups, showing no electrolyte abnormalities. The cholesterol plasmatic levels were similar for both formula groups. Additionally, both formula groups had similar blood protein patterns, with parallel levels of total proteins, albumin and prealbumin. The urea concentrations of the SYN1-supplemented infants were lower than those of the control group and more similar to those of the breastfed group. Few studies of infants fed with prebiotic-supplemented formulas have measured blood parameters. Alliet et al. identified no differences in lipid parameters (i.e., total cholesterol) between control and prebiotic-supplemented formula groups,[30] whereas others have noted no differences in total proteins, albumin and urea and only slight differences in prealbumin between control and prebiotic-supplemented formula groups.[31]

Considering all these studies, there is no evidence that prebiotic supplementation affects the biochemical parameters in the blood

and urine samples. For this reason, we can conclude that prebiotic supplementation in infant formula is safe.

A limitation of this study may be the relative high dropout rate. However, we believe that this high withdrawals proportion is usual in this kind of nutritional clinical trials in healthy infants. In order to compensate these drop-outs, additional participants were randomly recruited to achieve enough statistical power. However, the high number of study withdrawals undergone makes our results should be taken with relative caution.

Another possible limitation could be that infants could have been recruited during the first 4 weeks of life (being influenced by other type of feeding). However, as we did not find any difference at inclusion age between feeding groups and we did not find differences in distribution of infants recruited after hospital discharge, we think that the final results of our primary outcomes have not been affected. The *a posteriori* calculation of statistical power reveals that we would need 87 children per feeding group to find out differences about 11% in log cfu/g faeces of *Bifidobacterium*.

It was already proposed that 0.8 g/dL of SYN1 in formula milk might be a more effective dose than 0.4 g/dL of SYN1 and similar to FOS/GOS supplementation.[10] Our clinical trial demonstrates the safety and efficacy of this SYN1 0.8 g/dL supplemented formula. From a practical point of view, this clinical trial may support the use of this prebiotic in infant formulas.

In summary, this study demonstrates that SYN1 supplementation promotes a trend toward increased *Bifidobacterium* in the gut which might be the physiological mechanism to promote a deposition pattern closer to that promoted by human milk. Furthermore this softer stools pattern was not associated with any harmful effect such as disturbed growth or water balance. We conclude that 0.8 g/dL SYN1 supplementation in infant formula during the first 4 months of life is safe, effective and well tolerated.

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Statement of authorship

Closa-Monasterolo R carried out the Study design and coordination, data evaluation and writing of the manuscript. Gispert-Llaurado M carried out Field work, statistical analyses and writing of the manuscript. Luque V carried out the Study design, coordination of the field work, statistical analyses and writing of the manuscript. Ferre N carried out Coordination of the field work, statistical analyses and writing of the manuscript. Rubio-Torrents C carried out Field work and critical reading of the manuscript. Zaragoza-Jordana M carried out Study design and critical reading of the manuscript. Escribano J carried out the Study design and coordination, data evaluation and writing of the manuscript.

Conflict of interest

The authors have no conflicts of interest to declare.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.clnu.2013.02.009.

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9.5. INTENTION TO TREAT ANALYSES (ITT)

Figure 1. Participants included in the intention to treat analyses.

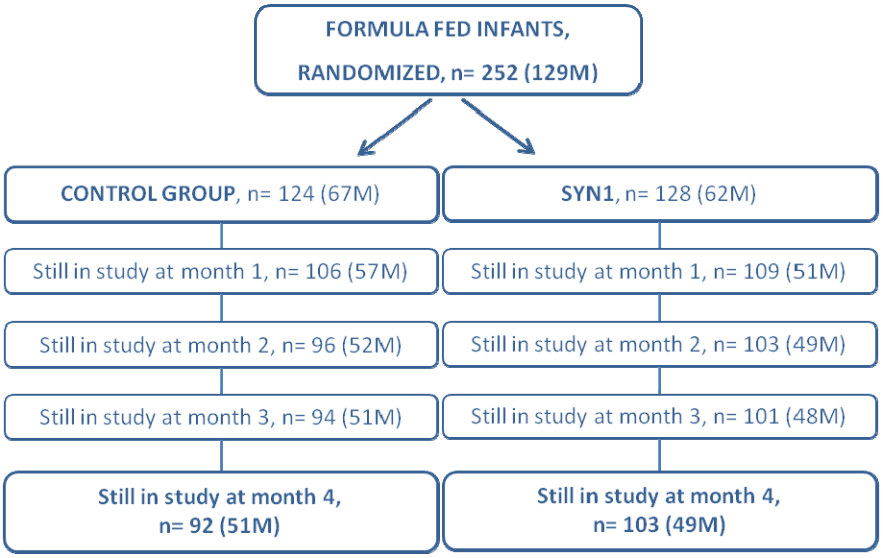

Table 1. Anthropometric characteristics of infants by feeding groups at all timepoints.

	Control Mean (SD)	SYN1 Mean (SD)	Control vs. SYN1 P value
MONTH 1	(n=106)	(n=109)	
Weight (g)	4175 (430)	4167 (440)	0.888
Length (cm)	53.8 (1.9)	54 (2)	0.430
Head circumference (cm)	37.3 (1.1)	37.2 (1.2)	0.672
Waist circumference (cm)	36.3 (2.1)	36.2 (2.1)	0.763
Arm circumference (cm)	11.3 (0.7)	11.3 (0.8)	0.835
Tricipital skinfold (mm)	6.4 (0.9)	6.2 (0.9)	0.067

	Control Mean (SD)	SYN1 Mean (SD)	Control vs. SYN1 P value
Subscapular skinfold	6.5 (1)	6.4 (1.1)	0.271
MONTH 2	(n=96)	(n=103)	
Weight (g)	5158 (526)	5159 (529)	0.991
Length (cm)	57.4 (1.9)	57.6 (2)	0.469
Head circumference (cm)	39 (1.1)	38.9 (1.2)	0.641
Waist circumference (cm)	39 (2.5)	39 (2.2)	0.849
Arm circumference (cm)	12.3 (0.9)	12.4 (0.8)	0.664
Tricipital skinfold (mm)	7.3 (1.2)	7.2 (1)	0.830
Subscapular skinfold	7.1 (1.2)	7.1 (1.3)	0.914
MONTH 3	(n=94)	(n=101)	
Weight (g)	6008 (619)	5978 (637)	0.739
Length (cm)	60.6 (1.9)	60.7 (2.1)	0.740
Head circumference (cm)	40.4 (1.1)	40.3 (1.3)	0.572
Waist circumference (cm)	40.7 (2.4)	40.7 (2.4)	0.904
Arm circumference (cm)	13.2 (0.8)	13.1 (0.8)	0.359
Tricipital skinfold (mm)	8.1 (1.4)	7.9 (1.3)	0.141
Subscapular skinfold	7.2 (1.1)	7.2 (1.2)	0.920
MONTH 4	(n=92)	(n=103)	
Weight (g)	6770 (716)	6698 (726)	0.486
Length (cm)	63.6 (1.9)	63.3 (2.4)	0.448
Head circumference (cm)	41.6 (1.2)	41.4 (1.3)	0.460
Waist circumference (cm)	41.6 (2.2)	41.8 (2.5)	0.477
Arm circumference (cm)	13.7 (0.8)	13.7 (0.8)	0.917
Tricipital skinfold (mm)	8.6 (1.5)	8.3 (1.4)	0.283
Subscapular skinfold	7.4 (1.3)	7.3 (1.3)	0.487

Table 2. Description of laboratory urinary parameters by feeding groups.

	Control	SYN1	Control vs.
	Median (IQR)	Median (IQR)	P value
MONTH 1	(n=73)	(n=70)	
Urinary Cl (mEq/L)	19 (11,30)	17 (10, 25.25)	0.178
Urinary K (mEq/L)	19.95	16 (10.58, 22.75)	0.052
Urinary Na (mEq/L)	17 (9.5, 26)	12 (7, 23)	0.065
Urinary Na/K ratio	0.85 (0.55,1.26)	0.85 (0.52,1.22)	0.691
Urinary Creatinine	8.5 (5.8,14.25)	8.4 (4.9,12.02)	0.143
Urinary Osmolarity	148 (94, 209.5)	115 (80,182)	0.122
MONTH 3	(n=65)	(n=68)	
Urinary Cl (mEq/L)	22 (10, 48.5)	19.5 (10.25, 31)	0.400
Urinary K (mEq/L)	19.8 (12.7, 32.9)	16.7 (10.53,	0.163
Urinary Na (mEq/L)	22 (7, 59.5)	16.5 (8, 40.75)	0.387
Urinary Na/K ratio	0.89 (0.44, 1.84)	1.08 (0.56, 1.54)	0.768
Urinary Creatinine	11.8 (6.5, 19.3)	8.9 (5.65, 16.45)	0.183
Urinary Osmolarity	159 (98, 269.5)	130.5 (73.75,	0.194

Table 3. Description of laboratory serum parameters by feeding groups.

	Control (n=34)	SYN1 (n=41)	Control vs. SYN1
	Mean (SD)	Mean (SD)	P value
MONTH 3			
Serum Cholesterol (mg/dL)	130.52 (15.79)	131.63 (20.46)	0.796
Serum Urea (mg/dL)	21.98 (3.58)	19.36 (3.63)	**0.002**
Serum Albumin (g/dL)	4.22 (0.36)	4.23 (0.39)	0.499
Serum Proteins (g/dL)	5.98 (0.36)	6.03 (0.41)	0.639

Serum Na (mEq/L)	138.6 (1.48)	138.49 (1.73)	0.790
Serum K (mEq/L)	5.48 (0.46)	5.44 (0.58)	0.748
Serum Cl (mEq/L)	105.24 (1.64)	106.02 (2.21)	0.095
Serum Prealbumin (mg/dL)	20.49 (7.51)	20.64 (8.96)	0.542
Serum pH	7.33 (0.05)	7.33 (0.05)	0.809
Serum base excess (mmol/L)	-1.41 (1.72)	-1.72 (2)	0.485
Serum Ca_ion (mmol/L)	1.33 (0.06)	1.31 (0.1)	0.361
IgG (mg/dL)	372.7 (106.9)	375.76 (120.25)	0.909
IgM (mg/dL)*	45.48 (1.62)	52.83 (1.44)	0.130
IgE (UI/mL)	10.88 (8.74)	10.95 (10.58)	0.985
IgG Diphteria (UI/mL)	0.22 (0.14)	0.31 (0.57)	0.711
IgG Tetanus (UI/mL)*	0.25 (2.48)	0.30 (2.81)	0.387

Table 4. Description of laboratory serum parameters by feeding groups.

	Control	SYN1	P value
	N (%)	N (%)	Control vs. SYN1
Serum C-reactive protein (over detection limit)			
Yes	1(2.9)	0 (0)	0.269
No	33 (97.1)	41 (100)	
IgG Diphteria cat (over detection limit)			
Yes	5 (17.2)	11 (29.7)	0.240
No	24 (82.8)	26 (70.3)	
IgE response (over detection limit)			
Yes	18 (54.5)	8 (20)	**0.002**
No	15 (45.5)	32 (80)	
IgG Pertusis (over detection limit)			
Yes	7 (21.9)	9 (23.1)	0.904
No	25 (78.1)	30 (76.9)	

Table 5. Description of digestive symptoms reported by parents (data from the parents' diary)

	Control	SYN1	P value
	Median (IQR)	Median (IQR)	Control vs. SYN1
MONTH 1	(n=101)	(n=109)	
Depositions (n)	2.5 (2, 4)	4 (2.5, 5)	**<0.001**
Regurgitation (n)	2 (0, 3.5)	1 (0, 3)	0.135
Vomits (n)	0 (0,0)	0 (0, 0)	0.784
Faecal consistency	6 (4.8, 6)	6 (6, 6)	**0.001**
MONTH 2	(n=94)	(n=101)	
Depositions (n)	2 (1.5, 3)	2.5 (2, 3.5)	**0.005**
Regurgitation (n)	1 (0, 3)	0.5 (0, 2.5)	0.504
Vomits (n)	0 (0, 0)	0 (0, 0)	0.859
Faecal consistency	6 (4.6, 6)	6 (6, 6)	**0.002**
MONTH 3	(n=93)	(n=98)	
Depositions (n)	2 (1.5, 2.5)	2 (1.5, 3.5)	**0.004**
Regurgitation (n)	0.5 (0, 2.3)	0.5 (0, 2.63)	0.956
Vomits (n)	0 (0,0)	0 (0,0)	0.182
Faecal consistency	6 (4, 6)	6 (6, 6)	**<0.001**
MONTH 4	(n=88)	(n=95)	
Depositions (n)	2 (1.5, 2.5)	2 (1.5, 3)	**0.044**
Regurgitation (n)	0.5 (0, 2)	0 (0, 2)	0.271
Vomits (n)	0 (0, 0)	0 (0, 0)	**0.042**
Faecal consistency	6 (4.1,6)	6 (6,6)	**<0.001**

Table 6. Mother's recall of usual digestive symptoms during the previous 4 weeks.

	Control n (%)	SYN1 n (%)	P value Control vs. SYN1
MONTH 1			
Frequent digestive discomfort			
Yes	39 (36.8)	30 (27.5)	0.146
No	67 (63.2)	79 (72.5)	
Frequent vomits			
Yes	1 (1)	5 (4.6)	0.107
No	104 (99)	104 (95.4)	
Frequent regurgitation			
Yes	10 (9.5)	15 (13.8)	0.335
No	95 (90.5)	94 (86.2)	
MONTH 2			
Frequent digestive discomfort			
Yes	22 (23.4)	18 (17.8)	0.335
No	72 (76.6)	83 (82.2)	
Frequent vomits			
Yes	9 (9.6)	15 (14.9)	0.114
No	85 (90.4)	86 (85.1)	
Frequent regurgitation			
Yes	2 (2.1)	7 (6.9)	0.262
No	92 (97.9)	95 (93.1)	
MONTH 3			
Frequent digestive discomfort			
Yes	2 (2.2)	5 (5)	0.297
No	90 (97.8)	95 (95)	
Frequent vomits			
Yes	13 (14)	12 (12.4)	0.197

	Control n (%)	SYN1 n (%)	P value Control vs. SYN1
No	80 (86)	85 (87.6)	
Frequent regurgitation			
Yes	1 (1.1)	4 (4)	0.743
No	93 (98.9)	96 (96)	
MONTH 4			
Frequent digestive discomfort			
Yes	1 (1.1)	3 (3)	0.354
No	90 (98.9)	96 (97)	
Frequent vomits			
Yes	8 (8.8)	10 (10.1)	0.343
No	83 (91.2)	89 (89.9)	
Frequent regurgitation			
Yes	1 (1.1)	3 (3.1)	0.758
No	91 (98.9)	95 (96.9)	