



Randomized Control Trials

Fermented infant formula (with *Bifidobacterium breve* C50 and *Streptococcus thermophilus* O65) with prebiotic oligosaccharides is safe and modulates the gut microbiota towards a microbiota closer to that of breastfed infants

Laurent Béghin^a, Sebastian Tims^{b,*}, Mieke Roelofs^b, Carole Rougé^c, Raish Oozeer^b, Thameur Rakza^d, Gaetano Chirico^e, Guus Roeselers^b, Jan Knol^{b,f}, Jean Christophe Rozé^g, Dominique Turck^{a,h}

^a Univ. Lille, CHU Lille, Clinical Investigation Center, CIC-1403–Inserm–CHU and U1286 - INFINITE - Institute for Translational Research in Inflammation, F-59000, Lille, France

^b Danone Nutricia Research, Utrecht, the Netherlands

^c Laboratoire Gallia, Limonest, France

^d Department of Neonatology, Lille University Jeanne de Flandre Children's Hospital and Faculty of Medicine, University of Lille, F-59000, Lille, France

^e Department of Neonatology, Spedali Civili, Brescia, Italy

^f Laboratory of Microbiology, Wageningen University, the Netherlands

^g Department of Neonatal Medicine, Nantes University Hospital, Nantes, France

^h Division of Gastroenterology, Hepatology and Nutrition, Department of Pediatrics, Lille University Jeanne de Flandre Children's Hospital and Faculty of Medicine, University of Lille, F-59000, Lille, France

ARTICLE INFO

Article history:

Received 25 November 2019

Accepted 17 July 2020

Keywords:

Healthy term infants

Prebiotics

Fermented formula

Postbiotics

Early-life microbiota

Secretory IgA

SUMMARY

Background & aims: Microbiome-modulators can help positively steer early-life microbiota development but their effects on microbiome functionality and associated safety and tolerance need to be demonstrated. We investigated the microbiome impact of a new combination of bioactive compounds, produced by the food-grade microorganisms *Bifidobacterium breve* C50 and *Streptococcus thermophilus* ST065 during a fermentation process, and prebiotics in an infant formula. Tolerance and safety were also assessed.

Methods: An exploratory prospective, randomized, double-blind, controlled, multi-centre study was designed to investigate the effect of bioactive compounds and prebiotics (short-chain galacto-oligosaccharides (scGOS)/long-chain fructo-oligosaccharides (lcFOS) 9:1). Experimental formulas containing these bioactive compounds and prebiotics (FERM/scGOS/lcFOS), prebiotics (scGOS/lcFOS), or bioactive compounds (FERM), were compared to a standard cow's milk-based control formula (Control). Exclusively breastfed infants were included as a reference arm since exclusive breastfeeding is considered as the optimal feeding for infants. The study lasted six months and included visits to health care professionals at baseline, two, four and six months of age. Stool SIgA concentration was the primary study outcome parameter.

Results: There were 280 infants randomized over the experimental arms and 70 infants entered the breastfed-reference arm. Demographics were balanced, growth and tolerance parameters were according to expectation and adverse events were limited. At four months of age the median SIgA concentration in the FERM/scGOS/lcFOS group was significantly higher compared to the Control group ($p = 0.03$) and was more similar to the concentrations found in the breastfed-reference group. *Bifidobacterium* increased over time in all groups. The FERM/scGOS/lcFOS combination resulted in a microbiota composition and metabolic activity closer to the breastfed infants' microbiome.

Conclusion: The FERM/scGOS/lcFOS combination showed a significant positive effect on SIgA levels. All formulas tested were associated with normal growth and were well-tolerated. Additionally, at four

* Corresponding author. Uppsalalaan 12, 3584 CT, Utrecht, the Netherlands.

E-mail address: sebastian.tims@danone.com (S. Tims).

months of age the FERM/scGOS/lcFOS formula brought the microbiome composition and metabolic activity closer towards that of breastfed infants.

Clinical trial registry: Registration number NTR2726 (Netherlands Trial Register; www.trialregister.nl/).
© 2020 The Authors. Published by Elsevier Ltd. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

1. Introduction

The human gut harbours a complex microbial ecosystem, the gut microbiota, which is known to enrich host functions related to the integrity of the gut lining [1], pathogen inhibition [2], functioning of the immune system [3], and energy homeostasis [4]. Several studies have demonstrated the important role the gut microbiota plays in the immune response and intestinal barrier function of the host [5,6]. Hence early life microbial colonization, which is in parallel and interdependent with the maturation of the gastrointestinal tract itself, is now considered as a critical step in healthy development [7]. This process is influenced by several environmental factors [8–10], among which early life nutrition is a key factor. The importance of early life nutrition is reflected in the microbiota composition; typically *Bifidobacterium* species dominate when infants are breastfed, while the microbiota composition consists of more members of the phylum Firmicutes when infants are fed a standard cow's milk based formula [11]. The World Health Organization (WHO) recommends exclusive breastfeeding until six months of age as, in early life, human milk is the optimal source of nutrition. However, due to various reasons, not all infants are able to be breastfed and, in these cases, infant formula is the source of early life nutrition.

Infant formulas now commonly contain microbiome modulators such as prebiotics and probiotics. According to the recent consensus statement of the International Scientific Association for Probiotics and Prebiotics (ISAPP), a prebiotic is defined as “a substrate that is selectively utilized by host microorganisms conferring a health benefit” [12]. Prebiotics are poorly digested and absorbed in the upper part of the gastrointestinal (GI) tract and most of them reach the colon unchanged. In the colon they serve as bacterial growth substrates and therefore they directly influence the composition and activity of the colonic microbiota. The characteristic microbiota composition and metabolic activity observed in full-term healthy breastfed infants is largely explained by the presence of oligosaccharides in human milk [13,14]. Several studies have shown that the microbiota of infants fed with formulas supplemented with a specific mixture of short chain galacto-oligosaccharides (scGOS) (90%) and long chain fructo-oligosaccharides (lcFOS) (10%) consists of higher levels of bifidobacteria compared to infants fed with a standard formula [15–18]. Also the metabolic activity of the gut microbiota of infants fed this prebiotic mixture has been shown to be closer to that of breastfed infants [19–22]. Moreover, as the addition of this prebiotic mixture leads to higher stool secretory IgA (SIgA) concentrations and lower infection rates [23–25] it is also suggested to influence intestinal immune development.

Fermentation is a way to generate postbiotics, which are defined here as bioactive compounds produced by food-grade microorganisms in a fermentation process (based on Aguilar-Toala and co-workers [26]). Such bioactive compounds, generated from the food matrix, provide a different strategy to modulate intestinal health in comparison to “probiotic” approaches in which living microorganisms are administered in adequate amounts as an ingredient. The bioactive compounds generated during a specific fermentation process that includes the combined activity of *Bifidobacterium breve*

C50 (BbC50) and *Streptococcus thermophilus* ST065 have been shown to have beneficial effects on the composition and metabolic activity of the intestinal microbiota in mice and humans, as indicated by an increase of faecal bifidobacteria levels, a decrease in the number of clostridia spores and *Bacteroides fragilis* counts and a lower stool pH [27–29]. Consumption of these bioactive compounds in healthy new-borns has been shown to be associated with enhanced production of intestinal SIgA antibodies to polioviruses after vaccine administration [29] and less severe diarrheal episodes compared to infants fed a standard formula [30,31]. The objective of this study was to explore if pairing these bioactive compounds with scGOS/lcFOS would lead to a beneficial effect on gut microbiota composition and metabolic activity as well as on immunological defence of the GI tract in early life. In addition, growth and safety were investigated.

2. Materials and methods

2.1. Study design

The effects of an infant formula containing both bioactive compounds (produced via a fermentation process) and prebiotics were investigated in healthy infants in this prospective, randomized, double-blind, four-arm parallel group, controlled, multi-centre study [Netherlands Trial Register: NTR2726]. Three experimental formulas either containing a combination of these bioactive compounds (FERM) and prebiotic oligosaccharides (FERM/scGOS/lcFOS), prebiotic oligosaccharides (scGOS/lcFOS), or the bioactive compounds (FERM) were compared to a standard cow's milk-based control formula. In addition, growth and safety were investigated. As a reference, healthy term infants who were exclusively breastfed during the first six months of life were assessed.

2.2. Subjects

This study investigated the effects of four infant formulas in healthy term infants. The inclusion criteria were: term birth (gestational age between 37 and 42 weeks); birth weight within normal ranges for gestational age and sex; fully formula-fed or had started the transition from breast-to formula-feeding; and aged ≤ 7 days. Infants were excluded due to the following reasons: in case of current and/or previous illnesses and/or congenital diseases or malformations in their mother or themselves that could interfere with the study (e.g., Group B streptococcal infection, severe diarrhoea), if they received antibiotics prior to baseline (directly or via breast-feeding); if they were at risk of developing allergy (family history of allergy defined as having at least one parent or blood-related sibling with clear atopic symptoms of hay fever, asthma or atopic dermatitis); if they consumed any formula that could interfere with the study prior to baseline; or if they needed a special diet other than standard cow's milk-based infant formula. Eligible infants were randomly assigned (1:1:1:1) to one of the treatment groups in blocks of 4 based on a computer-generated randomization sequence. Stratified randomization was performed using country as stratification factor. Infants who fit the above inclusion and exclusion criteria, who were exclusively breastfed since birth and whose mothers had the intention to continue exclusive breast-

feeding until the infant was at least four months of age were eligible for participation in the breastfed reference group.

2.3. Study formulas

Control and experimental products were powdered infant formulas providing complete nutritional support for infants in the first six months of life. The formulas were iso-caloric, and compositions were according to Directive 2006/141/EC (see Table 1). In total, three experimental formulas were tested: a standard cow's milk based infant formula containing bioactive compounds obtained by fermenting the base formula with *B. breve* C50 and *S. thermophilus* 065 using a specific fermentation process, and supplemented with a 90% short-chain galacto-oligosaccharides and 10% long-chain fructo-oligosaccharides prebiotic mixture (FERM/scGOS/lcFOS); a standard cow's milk based infant formula supplemented with the prebiotic mixture (9:1) only (scGOS/lcFOS); a standard cow's milk based infant formula containing these bioactive compounds only (FERM). During the fermentation process ST065 produces a major trisaccharide, which has been identified as D-galactopyranosyl-(1->3)-D-galactopyranosyl-(1->4)-D-glucose [32] (from now on referred to as 3'-GL). To maintain the 9:1 ratio between short-chain and long-chain oligosaccharides, scGOS was added at a concentration of 0.52 g/100 mL while 3'-GL was present in a concentration of 0.2 g/100 mL. The three investigational formulas were compared to a standard cow's milk based infant formula (Control) with the same composition as the investigational formulas, but without supplementation of prebiotic oligosaccharides or bioactive compounds. All formulas had a similar taste, smell, and colour and were manufactured according to Good Manufacturing Practices by Nutricia (Steenvoorde, France). The study products were stored at study sites in a secure and limited access storage area protected from extremes of light, temperature, and humidity.

2.4. Recruitment procedure and study conduction

The study recruited participants in three European countries (France, Germany, and Italy). Parents or guardians of infants that met the above inclusion and exclusion criteria and those who were exclusively breastfed since birth (with the intention to continue exclusive breast-feeding until the infant was at least four months of age) were approached in the first seven days of their infant's life. After obtaining written informed consent at the combined screening and baseline visit, the eligible infants were randomly assigned to one of the treatment groups or entered the breastfed reference group. A stool sample was collected prior to starting with the study formula. Further visits were conducted at two, four and

six months after birth. At each visit, stool samples and data on anthropometrics, feeding pattern, medication use, clinical symptoms and stool characteristics were collected by the infant's paediatrician. Two weeks after the last study visit a follow-up phone call was conducted, in which the infant's current feeding regimen, the occurrence of any adverse events, as well as related intake of medication and health care professional visits was recorded.

2.5. Growth-anthropometrics

Anthropometrical parameters (retrospectively collected for birth) were measured at each study visit by their paediatrician using standardized techniques. At each time point, for each growth parameter, three measurements were taken, and the average value was used. A SAS macro (provided by WHO, <http://www.who.int/childgrowth/software/en/>) was used to normalize the absolute anthropometric measurements of subjects, using the actual chronological age and sex of each subject.

2.6. Stool characteristics

Subjects' stool consistency was scored during the seven days before each visit, based on five categories: watery [1], loose [2], soft [3], formed [4] and hard [5].

2.7. Microbiota composition and stool metabolic activity

Stool parameters were analysed for infants who received no antibiotics during the study and who completed the study. These selected infants were further divided into two subgroups based on their mode of delivery, i.e., vaginally delivered infants and caesarean delivered infants. The bacterial composition of the stool microbiota was determined by Fluorescence In Situ Hybridization (FISH), a microscopic technique used for identification and counting of bacteria bacterial using specific 16S-rRNA targeted fluorescent DNA probes, as described previously [33]. The bacterial target groups and the associated probes used were: *Atopobium* cluster (Ato291; [11]); *Bacteroides distasonis* group (Bdis656; [34]); *Bacteroides fragilis* group (Bfra602; [34]); *Bifidobacterium* spp. (Bif164-mod; [35]); *Blautia coccooides* group (derived by subtracting Rrec584 measurements from Erec482 measurements); *Clostridium histolyticum* group (Chis150; [34]); *Clostridium lituseburense* group (Clit135; [34]); Subset Enterobacteriaceae (Ec1531; [36]); *Eubacterium rectale* and *B. coccooides* group (Erec482; [34]); *Lactobacillus* - *Enterococcus* group (Lab158; [37]); and the *Roseburia* and *E. rectale* group (Rrec584; [38]).

Table 1
Product composition used infant formulas.

		Per 100 mL			
		FERM/scGOS/lcFOS	scGOS/lcFOS	FERM	Control
Energy	kCal	67	67	68	69
Protein (g), of which		1.3	1.3	1.4	1.3
	Casein	0.7	0.5	1.0	0.7
	Whey protein	0.7	0.8	0.4	0.7
Carbohydrates (g), of which		7.3	7.3	8.6	8.1
	Sugar, of which	7.3	7.2	6.3	8.1
	Lactose	6.9	7.0	6.0	7.8
Fats (g), of which		3.5	3.5	3.1	3.5
	Linoleic acid (mg)	613	466	546	613
	α -linoleic acid (mg)	58	86	52	58
Fibre (g)		0.6	0.6	0.2	0

scGOS/lcFOS = short-chain galacto-oligosaccharides and long-chain fructo-oligosaccharides; FERM = fermented infant formula.

Additionally, the following targeted metabolic activity parameters were measured in the selected set of stool samples: pH; short chain fatty acid (SCFA) levels (i.e. acetate, propionate, butyrate, isobutyrate, valerate, and iso-valerate); D- and L-lactate; secretory immunoglobulin A (SIgA); and calprotectin. The methodologies to quantify these physiological parameters have been described in more detail previously [39].

2.8. Ethics

The study was registered with the Dutch Trial Register on February 1, 2011 (registration number NTR2726), and the approval of the relevant ethics committees in the participating countries was obtained before the start of the study. The study was conducted according to ICH-GCP principles, and in compliance with the principles of the 'Declaration of Helsinki' (2008) and with the local laws and regulations of each country where the study was performed.

2.9. Statistics

Sample size calculation was based on the expected difference in concentration of stool SIgA (primary outcome) between the FERM/scGOS/lcFOS and Control group (internal data). The required sample size for statistical testing using a significance level (α) of 0.05 and a power of 80% was 50 subjects per formula group. Allowing for a mixed feeding rate of 20% and a drop-out rate of 15%, a total of 280 (70 subjects per group) needed to be enrolled. A sample size of 70 subjects was considered sufficient to obtain reference data for the breast-fed reference group.

Testing for differences in SIgA concentrations between either of the experimental formula groups and the Control group was performed using ANOVA with the stratification factor 'country' as a covariate. Logarithmic transformation was applied before testing.

For the primary outcome, SIgA, a sensitivity analysis was run using multiple imputation. Missing values for SIgA were implemented using multiple imputation with 100 imputations. The Predictive Mean Matching Method was applied per visit using the variables "breastfed reference group versus randomized groups", "breastfeeding before randomization yes – no", "the mother's use of antibiotics during the last 15 days of pregnancy or during delivery", "use of prebiotics prior to randomization" and "mode of birth".

For the analysis of stool parameters, if < 30% of the values were below limit of detection (BLD) Wilcoxon rank sum tests (WR) were performed. If > 30% of the values were BLD, value BLD was replaced by "0" and the other values were replaced by "1," after which P values were calculated using Chi-Square test (Fisher's exact test in case of sparse cells) to assess differences in prevalence of the corresponding parameter.

In any other analyses, for continuous data, two sample t-tests were used. WRs were used in case of violation of normality assumption and/or presence of outliers. Categorical response parameters were analysed by using Chi-Square tests (Fisher's exact test in case of sparse cells). The statistical analysis was performed by Nutricia Research using SAS statistical software (SAS Enterprise Guide 4.3) for Windows (SAS Institute Inc., Cary, NC).

2.10. Randomization

The randomization sequence was generated using the PLAN procedure of SAS statistical software. The permuted block randomization was stratified for country, with a 1:1:1:1 allocation ratio of the four test products.

At the sponsor, the randomization sequence was saved electronically in a working environment with restricted access. Details about sequence generation, block size or sizes and whether the block size(s) were fixed or randomly were unknown to the investigators, site staff, and sponsor staff that were involved in the conduct of the study.

Based on the order in which subjects entered the study and the stratification factor, they were assigned a randomization number. The randomization number corresponded to the letter code of the study product that the subject was to receive (A, B, C or D).

The allocation sequence was generated by a statistician who was not involved in the conduct of the study. This statistician decided on the block size or sizes and whether the block size(s) were fixed or random. The sponsor's Clinical Study Supplies Manager was responsible for linking the randomisation numbers to the test and control products, assigning product codes to randomisation numbers, labelling the study products, creating randomisation envelopes and creating unblinding envelopes. The investigator was responsible for enrolling subjects into the study and assigning them to their given randomisation codes.

3. Results

3.1. Study population and randomization flow

Between February 2011 and April 2012, 350 subjects were enrolled in the study, of which 280 subjects were randomized to one of the four study formulas. The other 70 subjects were included in the breastfed reference group. At the end of the study, 197 of the randomized subjects completed the study, whereas 83 (30.3%) of the randomized subjects withdrew from the study prematurely. The most common reason for drop-out was an (serious) adverse event (AE) ($n = 39$). Other reasons were withdrawal of informed consent ($n = 10$), lost to follow-up ($n = 14$), protocol violation ($n = 11$), or other reasons ($n = 9$). The number of subjects that completed the study was not significantly different between the experimental groups or the control group. Subject flow is shown in Fig. 1.

3.2. Demographics and baseline characteristics

As a result of the randomization scheme applied in the study, subjects were well balanced over the study groups with respect to demographics and baseline characteristics. The observed differences were small and not considered clinically relevant. The most relevant characteristics are presented in SI Tables 1–4.

3.3. Primary outcome: SIgA

Stool SIgA concentrations are presented in Table 2 (for PP, vaginally born and caesarean born subgroups see SI Tables 5–7). At baseline, the median SIgA concentration in the stool samples of the four experimental formula groups, which were above detection limit, were very low (0.7 mcg/g faeces). In contrast, the baseline SIgA concentration in the breastfed group was very high (median 1860 mcg/g faeces) and gradually declined over time.

At 2 and 4 months of age the number of samples with SIgA concentrations above detection limit as well as the SIgA concentration was increased substantially in the four experimental groups (median 266–422 mcg/g faeces at 2 months of age; median 254–743 mcg/g faeces at 4 months of age). Notably, the median SIgA concentration in the FERM/scGOS/lcFOS group increased by more than double at the age of 4 months (743 mcg/g faeces) compared to the concentration at the age of 2 months (325 mcg/g faeces), thereby reaching the concentrations of SIgA in the breastfed reference group at the age of 4 months. The median SIgA

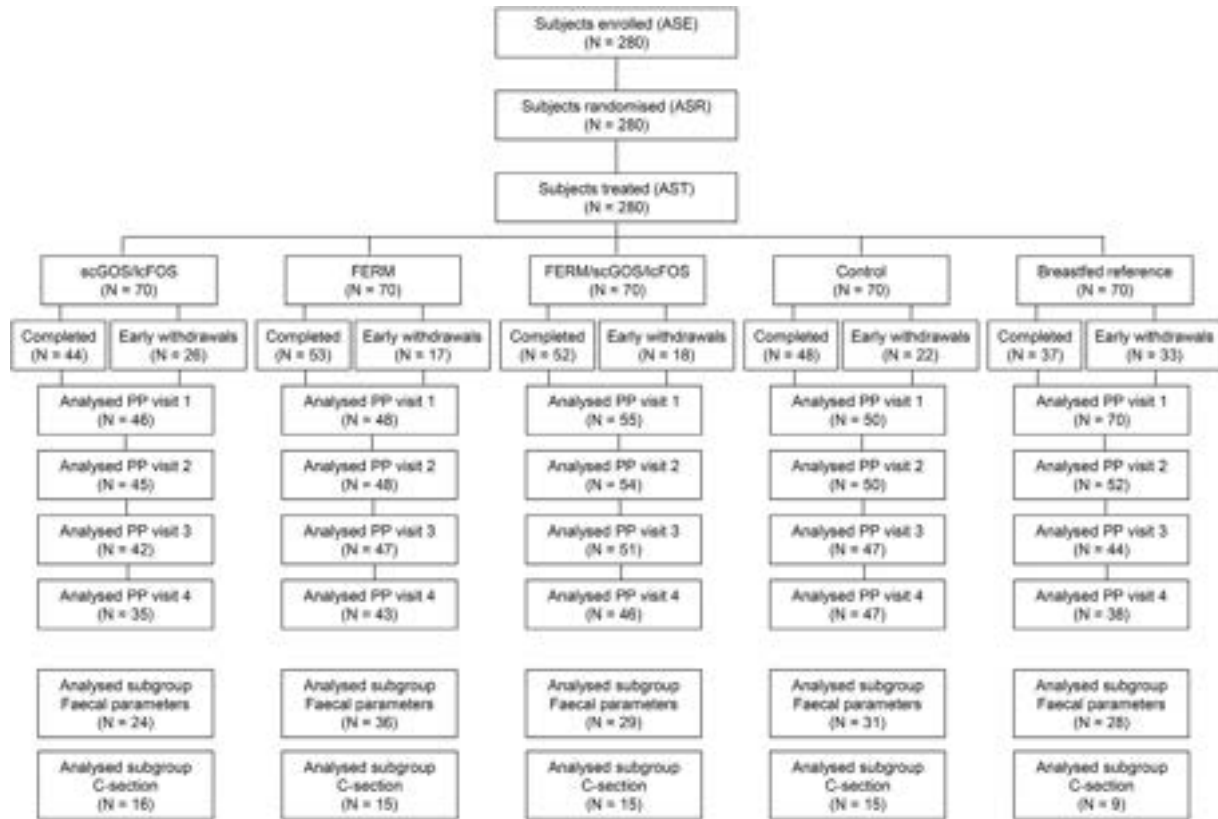


Fig. 1. Number of study subjects per study arm. ASE = all subjects enrolled; ASR = all subjects randomized; AST = all subjects treated; PP = per protocol; scGOS/lcFOS = short-chain galacto-oligosaccharides and long-chain fructo-oligosaccharides; FERM = fermented infant formula. ASR were included in the AST analysis and in the ITT analysis. The PP population was constructed by visit, so the number of subjects in the PP population differed per visit. Of the 280 subjects in the ITT at baseline, 199 subjects (71%) were included in the PP at baseline, 197 subjects (70%) at visit 2 (two months of age), 187 subjects at visit 3 (four months of age) and 171 subjects (61%) at visit 4 (six months of age). Of these, 120 subjects were included in a subgroup for stool parameter analysis and 61 subjects were included in a subgroup of infants born via caesarean section.

Table 2
Faecal SIgA concentrations (ITT).

Statistic		FERM/scGOS/lcFOS (N = 70)	scGOS/lcFOS (N = 70)	FERM (N = 70)	Control (N = 70)	Breastfed (N = 70)
SIgA	Baseline					
	Below LOD n (%)	50 (79.4%)	57 (90.5%)	55 (90.2%)	51 (83.6%)	1 (1.6%)
	Above LOD n (%)	13 (20.6%)	6 (9.5%)	6 (9.8%)	10 (16.4%)	60 (98.4%)
	Missing samples	7	7	9	9	9
	p-value ^a	0.646	0.293	0.422		
Month 2	Mean (SD)	903.3 (1247.7)	642.5 (915.5)	598.5 (709.9)	545.0 (541.0)	1158.6 (775.6)
	Median (Q1-Q3)	325.2 (192.5–1373.1)	421.6 (149.9–733.7)	265.6 (162.1–825.6)	403.6 (238.1–617.4)	948.3 (708.1–1535.5)
	Missing samples	16	20	19	22	20
	p-value ^b	0.488	0.630	0.695		
Month 4	Mean (SD)	929.4 (889.9)	698.6 (822.8)	539.0 (636.6)	685.3 (986.9)	1028.0 (603.0)
	Median (Q1-Q3)	742.9 (269.0–1517.8)	442.6 (142.3–854.1)	253.6 (120.7–756.7)	331.4 (154.5–663.5)	1051.7 (489.8–1443.5)
	Missing samples	23	27	21	29	33
	p-value ^b	0.028	0.789	0.360		
Month 6	Mean (SD)	872.4 (599.6)	723.6 (624.6)	608.1 (731.8)	735.6 (566.2)	1786.6 (1534.7)
	Median (Q1-Q3)	748.4 (501.6–1102.7)	609.4 (415.0–780.5)	423.5 (223.6–788.3)	615.2 (304.6–1015.3)	1642.0 (546.5–2401.2)
	Missing samples	30	32	31	36	32
	p-value ^b	0.478	0.586	0.218		

ITT = intention to treat; scGOS/lcFOS = short-chain galacto-oligosaccharides and long-chain fructo-oligosaccharides; FERM = fermented infant formula.

^a Pair-wise comparison between either of the investigational formulae groups and the Control group was done using Fisher's exact test.

^b Testing for differences between either of the investigational formulae groups and the Control group was done by using ANOVA with the stratification factor 'country' as a covariate. Logarithmic transformation was applied before testing.

concentration at 4 months of age in the FERM/scGOS/lcFOS group was significantly higher compared to the SIgA concentration in the Control group ($p = 0.03$).

The number of missing stool samples increased at the age of two, four and six months. Statistical analysis of the SIgA data using

multiple imputation of missing samples did not lead to relevant changes in the results. The median SIgA concentration at four months of age in the FERM/scGOS/lcFOS group was still significantly higher compared to the SIgA concentration in the Control group ($p = 0.04$).

3.4. Growth-anthropometrics

Results of the mean weight and length measurements per visit did not show any biologically relevant difference between either of the experimental formula groups, the Control group, or the breastfed reference group (see SI Tables 8–11). In comparison with the WHO Growth Standards [40], which are based on growth of exclusively breastfed infants, the mean values for weight, length and head circumference in all experimental groups were within the range of -1 to $+1$ s-scores (see Fig. 2).

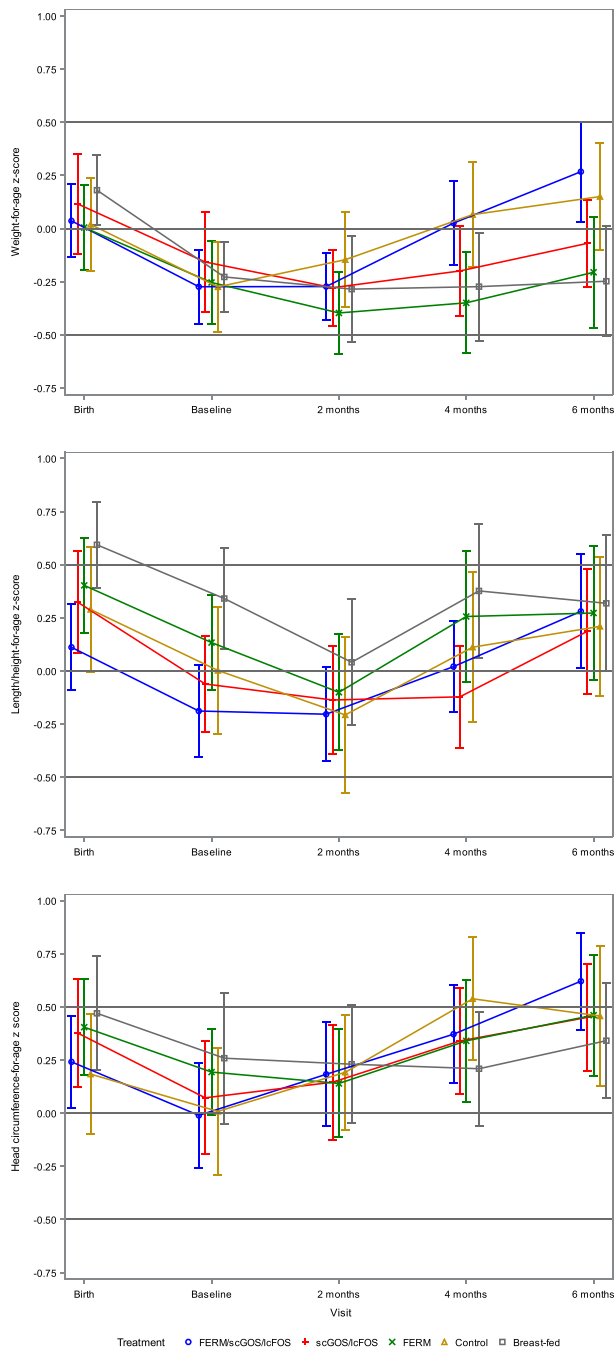


Fig. 2. Length/height-for-age (upper), weight-for-age (middle), and head circumference-for-length (lower) WHO z score: plot of predicted value plus 95% confidence limits for the ITT population per intervention group.

3.5. Stool characteristics

The average rank of stool consistency indicated a high incidence of loose and soft stools for all groups at two and four months of age, while the average rank shifted to a soft stool category for all groups at six months of age (see SI Table 8). At two and four months of age the stool consistency of the FERM/scGOS/lcFOS group was significantly softer compared to the Control group and was closer to that recorded in breastfed infants. These differences in stool consistency disappeared by the six months of age visit.

At four months of age, stool frequency in all experimental formula groups was significantly higher compared to the Control group. At two and six months of age stool frequency was not different between either of the experimental formula groups and the Control group (see SI Table 8). It is noteworthy that at two and four months of age, stool frequency of breastfed infants was higher compared to all groups receiving formula.

3.6. Safety outcomes

During the study a total number of 675 adverse events were reported in 188 subjects in the experimental formula groups and the Control group (see SI Table 8). The number of adverse events in the experimental formula groups did not statistically differ from the number of adverse events in the Control group. The majority of reported adverse events were considered mild and unrelated to the study product and were clustered in the following system organ classes: gastro-intestinal disorders (55.4%), infections (54.3%), and skin disorders (13.2%). More detailed information regarding the adverse events is provided in SI Tables 12–15.

3.7. Microbiota composition

The results of the microbiota composition analysis with FISH are shown in Fig. 3 and details are in SI Tables 16 and 17. In vaginally born subgroup there were no differences between experimental formulas for the following bacterial groups at any time point: the *Atopobium* cluster, the subset of Enterobacteriaceae, the *Lactobacillus - Enterococcus* group, and the *Roseburia* and *E. rectale* group (SI Table 16). These bacterial groups represent less than 10% of the infant microbiota measured here (highest levels at baseline). At baseline there was one bacterial group i.e., *Bacteroides fragilis* that showed a significant difference between study formulas. This difference, however, was not evident at two or four months of ages.

As expected [41] all study arms, including the breast-feeding reference group, showed a typical infant gut microbiota pattern, with a dominant presence of *Bifidobacterium* which increased over time. There were no differences across the formulas at baseline or at two months of age with respect to the percentage of *Bifidobacterium*, but there was a significant difference across the formulas ($p = 0.002$; Kruskal–Wallis test) at four months of age. This overall difference was mainly driven by the scGOS/lcFOS formula (see Fig. 3B–F). Similarly, for two potentially pathogenic bacterial groups, *Bacteroides distasonis* and *C. lituseburense*, and the more adult-like bacterial groups *E. rectale - B. coccoides* and the derived *B. coccoides*, there were no differences across the formulas at baseline or two months of age, but there were overall differences found across the formulas at four months of age ($p = 0.048$, $p < 0.001$, $p < 0.001$, $p < 0.001$, for the bacterial groups respectively; Kruskal–Wallis test). These differences were driven primarily by low abundance of these bacterial groups in the scGOS/lcFOS containing formulas (see Fig. 3).

There was no difference across formulas at baseline for *C. histolyticum*. However, significant differences across the formulas

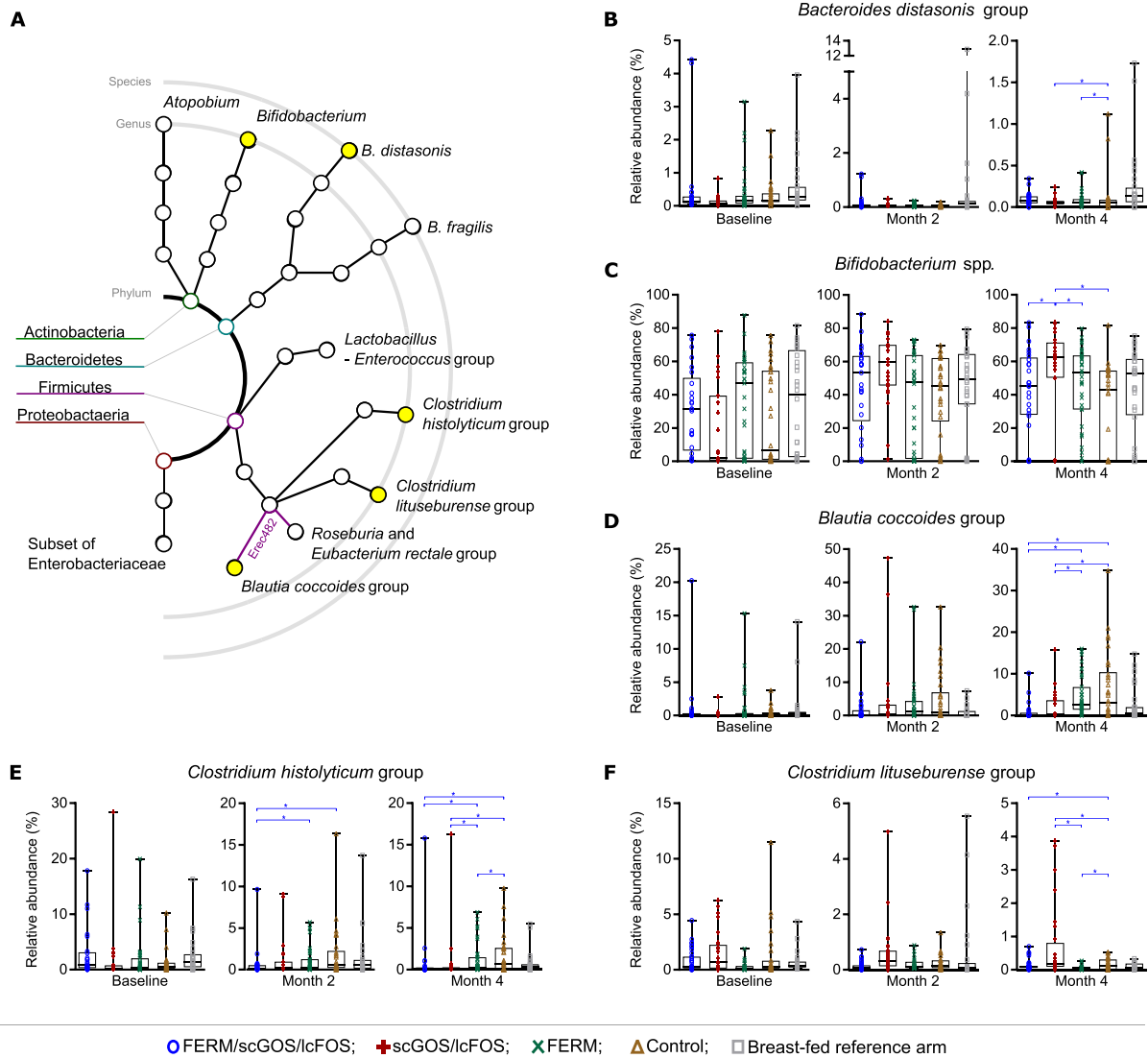


Fig. 3. Stool microbiota composition results. Panel A shows an overview of the phylogenetic relationship of all bacterial groups targeted by the Fluorescence In Situ Hybridization (FISH) assays on the stool microbiota composition. The yellow nodes indicate which bacterial groups showed significant differences across the study arms at two months and/or four months of age. Panel B–F show boxplots of the relative abundances (%) of these bacterial groups that showed significant differences. These significant differences are indicated by blue lines marked with an asterisk between the relevant study formulas. scGOS/lcFOS = short-chain galacto-oligosaccharides and long-chain fructo-oligosaccharides; FERM = fermented infant formula. Blue circles indicate FERM/scGOS/lcFOS, red pluses indicate scGOS/lcFOS, green crosses indicate FERM, brown triangles indicate Control, grey boxes indicate breastfed reference arm. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article).

at two and four months of age were detected ($p = 0.030$ and $p < 0.001$ respectively; Kruskal–Wallis test) (see Fig. 3E).

3.8. Stool metabolic activity

Baseline pH data showed no differences, but pH was significantly different between the formula groups at two and four months of age ($p < 0.001$ and $p < 0.001$, respectively; Kruskal–Wallis test) (see Table 3). Compared to Control, the median pH values were lower in the formulas containing prebiotics (FERM/scGOS/lcFOS and scGOS/lcFOS) at both two and four months of age. There was no significant difference in pH when comparing the FERM group to the Control group at any time points.

The number of stool samples with detectable amounts of the SCFAs butyric acid, iso-butyric acid, valeric acid, and iso-valeric acid and/or D-/L-lactate was low (see Table 3; SI Table 18). This limited quantitative analysis and a presence/absence analysis was

performed. No differences for these parameters were observed at baseline. For butyric acid, D-lactate, and L-lactate, significant differences between the formulas was observed at two months of age ($p = 0.006$, $p = 0.009$, $p = 0.040$, respectively; Chi–Square test). Butyric acid was not detected at all in the infants consuming FERM/scGOS/lcFOS, while D-lactate and L-lactate were detected in a significantly higher proportion of these when compared to those on the control formula.

At four months of age butyric acid remained significantly lower in FERM/scGOS/lcFOS formula fed infants compared to Control ($p = 0.001$; Chi–Square test). D-lactate and L-lactate were detected in a significantly higher proportion of the infants on both formulas that contained prebiotics (FERM/scGOS/lcFOS and scGOS/lcFOS) compared to Control ($p < 0.001$, $p = 0.001$, respectively for D-lactate; $p = 0.001$, $p = 0.004$, respectively for L-lactate; Chi–Square test). Iso-valeric acid was detected in a significantly lower proportion of the infants on the bioactive compounds and/or prebiotics

Table 3

Microbial metabolic activity parameters that showed significant differences across study formulas at any time point, vaginally born subgroup.

		Statistic	FERM/scGOS/lcFOSn = 29	scGOS/lcFOSn = 24	FERMn = 36	Control n = 31	Breastfedn = 28
Baseline	pH	Median (Q1–Q3) n (Nmiss)	5.6 (5.2–5.8) 29 (0)	5.8 (5.4–6.1) 23 (1)	5.8 (5.4–6.2) 36 (0)	5.4 (5.2–6.1) 30 (1)	5.6 (5.2–6.0) 26 (2)
	Organic acids						
	<i>Butyric Acid</i>	Detected n (%)	1 (4.5%)	2 (11.8%)	1 (3.3%)	1 (3.8%)	1 (5.3%)
	<i>Iso-Valeric Acid</i>	Detected n (%)	0 (0.0%)	1 (5.9%)	2 (6.7%)	2 (7.7%)	3 (15.8%)
	<i>D-lactate</i>	Detected n (%)	14 (63.6%)	8 (47.1%)	13 (43.3%)	13 (50.0%)	5 (26.3%)
	<i>L-lactate</i>	Detected n (%)	17 (77.3%)	6 (35.3%)	18 (60.0%)	17 (65.4%)	9 (47.4%)
		Missing	7	7	6	5	9
Month 2	pH ³	Median (Q1–Q3) n (Nmiss)	5.6 ¹ (5.4–6.3) 28 (1)	5.5 ¹ (5.3–6.3) 24 (0)	6.7 (6.1–7.4) 33 (3)	6.7 (5.9–6.9) 29 (2)	5.6 (5.3–5.9) 26 (2)
	Organic acids						
	<i>Butyric Acid</i>	Detected n (%)	0 (0.0%) ²	9 (39.1%)	13 (40.6%)	8 (28.6%)	3 (12.0%)
	<i>Iso-Valeric Acid</i>	Detected n (%)	4 (18.2%)	2 (8.7%)	8 (25.0%)	7 (25.0%)	6 (24.0%)
	<i>D-lactate</i>	Detected n (%)	16 (72.7%) ²	11 (47.8%)	11 (34.4%)	10 (35.7%)	13 (52.0%)
	<i>L-lactate</i>	Detected n (%)	15 (68.2%) ²	14 (60.9%)	10 (31.3%)	11 (39.3%)	18 (72.0%)
		Missing	7	1	4	3	3
Month 4	pH ³	Median (Q1–Q3) n (Nmiss)	5.4 ¹ (5.2–6.1) 26 (3)	5.9 ¹ (5.5–6.8) 20 (4)	6.7 (6.1–6.9) 34 (2)	6.9 (6.3–7.1) 22 (9)	5.6 (5.1–6.0) 27 (1)
	Organic acids						
	<i>Butyric Acid</i>	Detected n (%)	2 (9.1%) ²	6 (31.6%)	14 (42.4%)	12 (57.1%)	4 (23.5%)
	<i>Iso-Valeric Acid</i>	Detected n (%)	2 (9.1%) ²	0 (0.0%) ²	8 (24.2%) ²	12 (57.1%)	1 (5.9%)
	<i>D-lactate</i>	Detected n (%)	17 (77.3%) ²	14 (73.7%) ²	9 (27.3%)	4 (19.0%)	5 (29.4%)
	<i>L-lactate</i>	Detected n (%)	18 (81.8%) ²	14 (73.7%) ²	10 (30.3%)	6 (28.6%)	10 (58.8%)
		Missing	7	5	3	10	11

scGOS/lcFOS = short-chain galacto-oligosaccharides and long-chain fructo-oligosaccharides; FERM = fermented infant formula.¹Median values that, based on Wilcoxon Rank Sum test, are significantly different ($p < 0.05$) from the median of the Control group. Test performed if Kruskal–Wallis test comparing all treatment groups (excluding breastfed reference group) was significant.²Significant difference ($p < 0.05$) compared to the Control group, based on the Fisher's exact test or Chi–Square test; if there is at least one cell with expected count < 6 , Fisher's exact test was used. Otherwise, the Chi–Square test was used.³Significant ($p < 0.05$) Kruskal–Wallis test comparing all treatment groups (excluding breastfed reference group). Overall comparison is only done for continuous (and not binary) data.

containing formulae (FERM/scGOS/lcFOS, scGOS/lcFOS, and FERM) compared to Control ($p = 0.001$, $p < 0.001$, $p = 0.015$, respectively; Chi–Square test) at four months of age.

4. Discussion

One of the benefits of exclusive breastfeeding, the optimal mode of nutrition in infancy, is a high level of SIgA in the gastrointestinal tract which is not observed in infants on standard cow's milk formula. This study showed that the combination of bioactive compounds, produced by *B. breve* C50 and *S. thermophilus* O65 during a fermentation process, and prebiotics led to a significant increase on stool SIgA concentrations, the primary outcome of this study. Concomitant with this positive effect on SIgA concentrations, the combination of these bioactive compounds and prebiotics was shown to lower the stool pH compared to both control formula and to the formula containing the bioactive compounds alone. The combination was also shown to increase bifidobacteria and affect microbial metabolites as previously described for formulas with scGOS/lcFOS (9:1). In addition, we observed a combination-specific influence on the overall microbial composition, which steered early microbial colonization closer to what is observed in exclusively breastfed infants. Furthermore, infants fed with this combined formula grew well and the formula was well-tolerated.

The primary outcome SIgA, which, as a part of the gut-associated lymphoid tissue (GALT), plays a significant role in the immunological defence of the GI tract, serves as a first line of defence in protecting the intestinal epithelium from enteric toxins and pathogenic microorganisms. It has been widely demonstrated that production of SIgA results from interaction between the intestinal immune system and bacterial colonization [42]. Since neonates are born without the capacity to synthesize SIgA themselves, and maternal SIgA is likely washed out of the infant gut within two days after the cessation of breastfeeding, it was considered unlikely that breast-feeding prior to randomization

into this study would influence SIgA concentrations at two months of age and beyond [43]. For this reason, any breast-feeding before study entry was not considered in the analyses of the SIgA data. The four months' time-point was of key interest as prior to this age an infant's production of SIgA is still developing and after four months of age a high proportion of the participating infants were no longer exclusively formula-fed, due to the introduction of complementary foods. As expected, the highest levels of stool SIgA were found in breast-fed infants at four months of age. Previous studies reported higher stool SIgA concentrations in infants fed a formula supplemented with scGOS/lcFOS than in infants fed a standard formula [23,24], confirming that stool SIgA stimulation is possible by these prebiotics alone. At four months of age stool SIgA in the study group consuming the combined bioactive compounds and prebiotics formula (FERM/scGOS/lcFOS) was significantly higher compared to both the Control and formula with bioactive compounds only (FERM) groups. As scGOS/lcFOS alone did not show an effect of similar magnitude, these results suggest an additive effect of the combination of bioactive compounds and prebiotics as compared to either component alone. The mechanism behind this additive effect remains to be determined. These bioactive compounds have been shown to enhance the reactivity of the infant immune system by increasing production of intestinal SIgA antibodies to polioviruses following vaccine administration [30]. Additionally, one of the bioactive compounds produced by ST065 is 3'-GL [32], which is also present in human milk, in concentrations known to fluctuate during the different stages of lactation [44]. Oligosaccharides, especially those that are high in colostrum such as 3'-GL, appear to be able to modulate the immune system by affecting the Th1:Th2 cell ratio [44]. Therefore, the 3'-GL addition via fermentation is hypothesized to offer an oligosaccharide profile closer to the galactosyl lactose structures found in human milk.

Previous studies have shown that infants fed an infant formula supplemented with the prebiotic scGOS/lcFOS mixture have higher levels of bifidobacteria and lactobacilli compared to infants fed

standard formula [15–18], and an overall metabolic profile that appears closer to that of breastfed infants [19–22]. The latter is demonstrated via an acidic pH, an acetate dominant SCFA spectrum, and higher levels of lactate. Even if there were no differences in acetate levels, the lower pH levels and the lower butyrate and iso-valerate occurrence, as well as the higher lactate occurrence in the study formulas with prebiotics (FERM/scGOS/lcFOS and scGOS/lcFOS) compared to the Control formula, confirms that the beneficial properties of the prebiotics are maintained with the addition of the bioactive compounds. These results are in line with those previously reported for a combination of bioactive compounds and prebiotics derived from the same strains using the Lactofidus™ fermentation process; in this case the prebiotic effects on the stool microbiota were reported to be maintained [39].

In addition to the *Bifidobacterium* spp., other bacterial groups responded to the specific interventions as well. Both bioactive compounds and prebiotics seemed to repress the abundance of the *Bacteroides distasonis* (recently renamed as *Parabacteroides distasonis* [45]), the *C. lituseburensis*, and the *C. histolyticum* groups, with this most evident at four months of age. Although these groups are interesting, as previous studies link their presence to opportunistic pathogenic properties such as infections, enterotoxin production, and other unhealthy states [46,47], their abundances were even lower compared to the breastfed reference arm. More substantial differences were observed for the *B. coccoides* group. The combination of bioactive compounds and prebiotics (FERM/scGOS/lcFOS) particularly showed a repression of this taxon that was closer to the range observed in the breastfed reference arm. Combining the prebiotic scGOS/lcFOS mixture and bioactive compounds produced by BbC50 and ST065 in other independent trials has also been shown to reduce *Blautia* levels [48]. Members of the genus *Blautia* have been shown to possess specific acetogenic pathways coupled to hydrogen (H₂) oxidation [49,50]. H₂ is a general by-product of colonic bacterial metabolism. Given that the effect on *Blautia* is to bring the microbiota composition closer to that observed in breastfed infants, we hypothesize that *Blautia* levels in infants is a marker for H₂ status and that the lower levels of *Blautia* observed in infants consuming the FERM/scGOS/lcFOS mixture could possibly reflect or contribute to a healthier or more balanced microbial ecosystem.

Growth patterns in all study groups were according expectations (based on WHO standards). There was no difference in the prevalence of reported adverse events between the experimental formula groups and the Control group. Frequency and types of reported adverse events did not raise safety concerns. Analyses of the tolerance data showed a shift towards softer stools in infants consuming the prebiotics containing formulas (FERM/scGOS/lcFOS and scGOS/lcFOS) at four months of age. This reduction in consistency remained within the normal physiological ranges (based on observations in the breastfed reference group). Hence, the study formulas were considered well-tolerated and safe based on growth and adverse events outcomes.

In conclusion, the combination of bioactive compounds, produced by *B. breve* C50 and *S. thermophilus* ST065 during a fermentation process, and prebiotics in infant formula showed a significant positive effect on stool SIgA concentrations. All formulas tested in this study were associated with normal growth and were well-tolerated; limited adverse events were identified. At four months of age, the formula containing the combination of bioactive compounds and prebiotics brought the microbiota composition and metabolic activity towards that of term-born breastfed infants. These changes were associated with the higher SIgA levels but a mechanism of action for this effect requires further investigation. We demonstrate that this experimental infant formula containing bioactive compounds, produced by strains BbC50 and ST065,

combined with prebiotics emulates part of the gut metabolic activity observed in exclusively breastfed infants born at term.

Statement of authorship

RO, CR, and JK designed research; LB and TR conducted research; ST and MR analysed data or performed statistical analysis; LB, ST, MR, GR, and DT wrote paper; JK, JCR, GC, and DT had primary responsibility for final content.

Funding sources

Authors ST, MR, RO, GR, and JK are employees of Danone Nutricia Research which funded the Clinical Trial. RO and JK are inventors of patents WO 2017/114900 and WO 2017/114901 but are not right holders.

Conflict of Interest

Authors LB, TR, GC, JCR, and DT have no potential conflicts of interest.

Acknowledgements

The authors would like to thank all the families who participated in the study, as well as all the participating paediatricians from France (M. Bricout, J.F. Lienhardt, J.C. Requillart, V. Fournier, J. Stagnara, P. Robiliard, J.P. Bernet, S. Moore, F. Jeannerot, C. Fassler, C. Tohier, F. Vulser, and J. Gemellie) and Germany (C. Grüber [post-humously], S. Mirosław, and P. Kroschwald) and the research staff for their contribution to the study. We also would like to thank the French Association of Ambulatory Paediatrics for supporting this study, Annie Plé from CIC-1403-Inserm-CHU for logistical assistance and Dr Patrick Gelé from CRB-CIC-1403-Inserm-CHU for biological sample storage management and traceability. The authors would like to thank Tiemen van Eijndthoven for the molecular analysis on the stool samples.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.clnu.2020.07.024>.

References

- [1] Natividad JM, Verdu EF. Modulation of intestinal barrier by intestinal microbiota: pathologic and therapeutic implications. *Pharmacol Res* 2013;69(1): 42–51. <https://doi.org/10.1016/j.phrs.2012.10.007>.
- [2] Baumler AJ, Sperandio V. Interactions between the microbiota and pathogenic bacteria in the gut. *Nature* 2016;535(7610):85–93. <https://doi.org/10.1038/nature18849>.
- [3] Gensollen T, Iyer SS, Kasper DL, Blumberg RS. How colonization by microbiota in early life shapes the immune system. *Science* 2016;352(6285):539–44. <https://doi.org/10.1126/science.aad9378>.
- [4] den Besten G, van Eunen K, Groen AK, Venema K, Reijngoud DJ, Bakker BM. The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. *J Lipid Res* 2013;54(9):2325–40. <https://doi.org/10.1194/jlr.R036012>.
- [5] Sudo N, Sawamura S, Tanaka K, Aiba Y, Kubo C, Koga Y. The requirement of intestinal bacterial flora for the development of an IgE production system fully susceptible to oral tolerance induction. *J Immunol* 1997;159(4):1739–45. <https://doi.org/10.1111/pai.12232>.
- [6] Round JL, Mazmanian SK. The gut microbiota shapes intestinal immune responses during health and disease. *Nat Rev Immunol* 2009;9(5):313–23. <https://doi.org/10.1038/nri2515>.
- [7] Wopereis H, Oozeer R, Knipping K, Belzer C, Knol J. The first thousand days - intestinal microbiology of early life: establishing a symbiosis. *Pediatr Allergy Immunol* : Off Pub Eur Soc Pediatr Allergy Immuno 2014;25(5):428–38. <https://doi.org/10.1111/pai.12232>.
- [8] Boehm G, Jelinek J, Stahl B, van Laere K, Knol J, Fanaro S, et al. Prebiotics in infant formulas. *J Clin Gastroenterol* 2004;38(6 Suppl):S76–9.

- [9] Gueimonde M, Sakata S, Kalliomaki M, Isolauri E, Benno Y, Salminen S. Effect of maternal consumption of lactobacillus GG on transfer and establishment of fecal bifidobacterial microbiota in neonates. *J Pediatr Gastroenterol Nutr* 2006;42(2):166–70. <https://doi.org/10.1097/01.mpg.0000189346.25172.f0>.
- [10] Penders J, Thijs C, Vink C, Stelma FF, Snijders B, Kummeling I, et al. Factors influencing the composition of the intestinal microbiota in early infancy. *Pediatrics* 2006;118(2):511–21. <https://doi.org/10.1542/peds.2005-2824>.
- [11] Harmsen HJ, Wildeboer-Veloo AC, Raangs GC, Wagendorp AA, Klijn N, Bindels JG, et al. Analysis of intestinal flora development in breast-fed and formula-fed infants by using molecular identification and detection methods. *J Pediatr Gastroenterol Nutr* 2000;30(1):61–7.
- [12] Gibson GR, Hutkins R, Sanders ME, Prescott SL, Reimer RA, Salminen SJ, et al. Expert consensus document: the International Scientific Association for Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of prebiotics. *Nat Rev Gastroenterol Hepatol* 2017;14(8):491–502. <https://doi.org/10.1038/nrgastro.2017.75>.
- [13] Coppa GV, Zampini L, Galeazzi T, Gabrielli O. Prebiotics in human milk: a review. *Dig Liver Dis* 2006;38(Suppl 2):S291–4. [https://doi.org/10.1016/S1590-8658\(07\)60013-9](https://doi.org/10.1016/S1590-8658(07)60013-9).
- [14] Boehm G, Stahl B. Oligosaccharides from milk. *J Nutr* 2007;137(3 Suppl 2):847S–9S.
- [15] Decsi T, Arato A, Balogh M, Dolinay T, Kanjo AH, Szabo E, et al. [Randomised placebo controlled double blind study on the effect of prebiotic oligosaccharides on intestinal flora in healthy infants]. *Orv Hetil* 2005;146(48):2445–50.
- [16] Moro G, Minoli I, Mosca M, Fanaro S, Jelinek J, Stahl B, et al. Dosage-related bifidogenic effects of galacto- and fructooligosaccharides in formula-fed term infants. *J Pediatr Gastroenterol Nutr* 2002;34(3):291–5.
- [17] Haarman M, Knol J. Quantitative real-time PCR analysis of fecal Lactobacillus species in infants receiving a prebiotic infant formula. *Appl Environ Microbiol* 2006;72(4):2359–65. <https://doi.org/10.1128/AEM.72.4.2359-2365.2006>.
- [18] Haarman M, Knol J. Quantitative real-time PCR assays to identify and quantify fecal Bifidobacterium species in infants receiving a prebiotic infant formula. *Appl Environ Microbiol* 2005;71(5):2318–24. <https://doi.org/10.1128/AEM.71.5.2318-2324.2005>.
- [19] Boehm G, Lidestri M, Casetta P, Jelinek J, Negretti F, Stahl B, et al. Supplementation of a bovine milk formula with an oligosaccharide mixture increases counts of faecal bifidobacteria in preterm infants. *Arch Dis Child Fetal Neonatal Ed* 2002;86(3):F178–81.
- [20] Moro G, Arslanoglu S, Stahl B, Jelinek J, Wahn U, Boehm G. A mixture of prebiotic oligosaccharides reduces the incidence of atopic dermatitis during the first six months of age. *Arch Dis Child* 2006;91(10):814–9. <https://doi.org/10.1136/adc.2006.098251>.
- [21] Schmelzle H, Wirth S, Skopnik H, Radke M, Knol J, Bockler HM, et al. Randomized double-blind study of the nutritional efficacy and bifidogenicity of a new infant formula containing partially hydrolyzed protein, a high beta-palmitic acid level, and nondigestible oligosaccharides. *J Pediatr Gastroenterol Nutr* 2003;36(3):343–51.
- [22] Knol J, Scholtens P, Kafka C, Steenbakkers J, Gro S, Helm K, et al. Colon microflora in infants fed formula with galacto- and fructo-oligosaccharides: more like breast-fed infants. *J Pediatr Gastroenterol Nutr* 2005;40(1):36–42.
- [23] Scholtens PA, Alliet P, Raes M, Alles MS, Kroes H, Boehm G, et al. Fecal secretory immunoglobulin A is increased in healthy infants who receive a formula with short-chain galacto-oligosaccharides and long-chain fructo-oligosaccharides. *J Nutr* 2008;138(6):1141–7. <https://doi.org/10.1093/jn/138.6.1141>.
- [24] Bakker-Zierikzee AM, Alles MS, Knol J, Kok FJ, Tolboom JJ, Bindels JG. Effects of infant formula containing a mixture of galacto- and fructo-oligosaccharides or viable Bifidobacterium animalis on the intestinal microflora during the first 4 months of life. *Br J Nutr* 2005;94(5):783–90. S0007114505002473 [pii].
- [25] Bruzzese E, Volpicelli M, Salvini F, Bisceglia M, Lionetti P, Cinquetti M, et al. Early administration OF GOS/FOS prevents intestinal and respiratory infections IN infants. *J Pediatr Gastroenterol Nutr* 2006;42(5):E95.
- [26] Aguilar-Toalá JE, García-Varela R, García HS, Mata-Haro V, González-Córdova AF, Vallejo-Cordoba B, et al. Postbiotics: an evolving term within the functional foods field. *Trends Food Sci Technol* 2018;75:105–14. <https://doi.org/10.1016/j.tifs.2018.03.009>.
- [27] Romond MB, Ais A, Guillemot F, Bounouader R, Cortot A, Romond C. Cell-free whey from milk fermented with Bifidobacterium breve C50 used to modify the colonic microflora of healthy subjects. *J Dairy Sci* 1998;81(5):1229–35. [https://doi.org/10.3168/jds.S0022-0302\(98\)75683-8](https://doi.org/10.3168/jds.S0022-0302(98)75683-8).
- [28] Romond MB, Ais A, Yazourh A, Romond C. Cell-free wheys from bifidobacteria fermented milks exert a regulatory effect on the intestinal microflora of mice and humans. *Anaerobe* 1997;3(2–3):137–43. <https://doi.org/10.1006/anae.1997.0090>.
- [29] Mullie C, Yazourh A, Thibault H, Odou MF, Singer E, Kalach N, et al. Increased poliovirus-specific intestinal antibody response coincides with promotion of Bifidobacterium longum-infantis and Bifidobacterium breve in infants: a randomized, double-blind, placebo-controlled trial. *Pediatr Res* 2004;56(5):791–5. <https://doi.org/10.1203/01.PDR.0000141955.47550.AO>.
- [30] Indrio F, Ladisa G, Mautone A, Montagna O. Effect of a fermented formula on thymus size and stool pH in healthy term infants. *Pediatr Res* 2007;62(1):98–100. <https://doi.org/10.1203/pdr.0b013e31806772d3>.
- [31] Thibault H, Aubert-Jacquin C, Goulet O. Effects of long-term consumption of a fermented infant formula (with Bifidobacterium breve c50 and Streptococcus thermophilus 065) on acute diarrhea in healthy infants. *J Pediatr Gastroenterol Nutr* 2004;39(2):147–52.
- [32] Perrin V, Fenet B, Praly JP, Lecroix F, Ta CD. Identification and synthesis of a trisaccharide produced from lactose by transgalactosylation. *Carbohydr Res* 2000;325(3):202–10.
- [33] van der Aa LB, Heymans HS, van Aalderen WM, Sillevius Smitt JH, Knol J, Ben Amor K, et al. Effect of a new synbiotic mixture on atopic dermatitis in infants: a randomized-controlled trial. *Clin Exp Allergy* 2010;40(5):795–804. <https://doi.org/10.1111/j.1365-2222.2010.03465.x>.
- [34] Franks AH, Harmsen HJ, Raangs GC, Jansen GJ, Schut F, Welling GW. Variations of bacterial populations in human feces measured by fluorescent in situ hybridization with group-specific 16S rRNA-targeted oligonucleotide probes. *Appl Environ Microbiol* 1998;64(9):3336–45.
- [35] Langendijk PS, Schut F, Jansen GJ, Raangs GC, Kamphuis GR, Wilkinson MH, et al. Quantitative fluorescence in situ hybridization of Bifidobacterium spp. with genus-specific 16S rRNA-targeted probes and its application in fecal samples. *Appl Environ Microbiol* 1995;61(8):3069–75.
- [36] Poulsen LK, Licht TR, Rang C, Krogfelt KA, Molin S. Physiological state of Escherichia coli B4 growing in the large intestines of streptomycin-treated mice. *J Bacteriol* 1995;177(20):5840–5.
- [37] Harmsen HJ, Elferich P, Schut F, Welling GW. A 16S rRNA-targeted probe for detection of lactobacilli and enterococci in faecal samples by fluorescent in situ hybridization. *Microb Ecol Health Dis* 1999;11(1):3–12. <https://doi.org/10.1080/089106099435862>.
- [38] Walker AW, Duncan SH, McWilliam Leitch EC, Child MW, Flint HJ. pH and peptide supply can radically alter bacterial populations and short-chain fatty acid ratios within microbial communities from the human colon. *Appl Environ Microbiol* 2005;71(7):3692–700. <https://doi.org/10.1128/AEM.71.7.3692-3700.2005>.
- [39] Huet F, Abrahamse-Berkeveld M, Tims S, Simeoni U, Beley G, Savagner C, et al. Partly fermented infant formulae with specific oligosaccharides support adequate infant growth and are well-tolerated. *J Pediatr Gastroenterol Nutr* 2016;63(4):e43–53. <https://doi.org/10.1097/MPG.0000000000001360>.
- [40] Group WHOMGRS. WHO Child Growth Standards based on length/height, weight and age. *Acta Paediatr* 2006;450:76–85.
- [41] Oozeer R, van Limpt K, Ludwig T, Ben Amor K, Martin R, Wind RD, et al. Intestinal microbiology in early life: specific prebiotics can have similar functionalities as human-milk oligosaccharides. *Am J Clin Nutr* 2013;98(2). <https://doi.org/10.3945/ajcn.112.038893>. 561S–71S.
- [42] Cindy G, Giuliana M, Andrea C. Intestinal IgA production and its role in host-microbe interaction. *Immunol Rev* 2014;260(1):76–85. <https://doi.org/10.1111/immr.12189>.
- [43] Kawano A, Emori Y. Changes in maternal secretory immunoglobulin A levels in human milk during 12 weeks after parturition. *Am J Hum Biol* 2013;25(3):399–403. <https://doi.org/10.1002/ajhb.22387>.
- [44] He Y, Liu S, Leone S, Newburg DS. Human colostrum oligosaccharides modulate major immunologic pathways of immature human intestine. *Mucosal Immunol* 2014;7(6):1326–39. <https://doi.org/10.1038/mi.2014.20>.
- [45] Sakamoto M, Benno Y. Reclassification of Bacteroides distasonis, Bacteroides goldsteinii and Bacteroides merdae as Parabacteroides distasonis gen. nov., comb. nov., Parabacteroides goldsteinii comb. nov. and Parabacteroides merdae comb. nov. *Int J Syst Evol Microbiol* 2006;56(Pt 7):1599–605. <https://doi.org/10.1099/ijs.0.64192-0>.
- [46] Wexler HM. Bacteroides: the Good, the bad, and the nitty-gritty. *Clin Microbiol Rev* 2007;20(4):593–621. <https://doi.org/10.1128/CMR.00008-07>.
- [47] Gerritsen J, Smidt H, Rijkers GT, de Vos WM. Intestinal microbiota in human health and disease: the impact of probiotics. *Gene Nutr* 2011;6(3):209–40. <https://doi.org/10.1007/s12263-011-0229-7>.
- [48] Tims S, Roeselers G, FIPS&LIFE-study-group, Knol J. Gut microbiota composition modulation by partly fermented infant formulae supplemented with prebiotics scGOS/lcFOS. Geneva: ESPGHAN; 2018.
- [49] Bernalier A, Willems A, Leclerc M, Rochet V, Collins MD. Ruminococcus hydrogenotrophicus sp. nov., a new H₂/CO₂-utilizing acetogenic bacterium isolated from human feces. *Arch Microbiol* 1996;166(3):176–83. <https://doi.org/10.1007/s002030050373>.
- [50] Liu C, Finegold SM, Song Y, Lawson PA. Reclassification of Clostridium coccoides, Ruminococcus henseni, Ruminococcus hydrogenotrophicus, Ruminococcus luti, Ruminococcus productus and Ruminococcus schinkii as Blautia coccoides gen. nov., comb. nov., Blautia henseni comb. nov., Blautia hydrogenotrophica comb. nov., Blautia luti comb. nov., Blautia producta comb. nov., Blautia schinkii comb. nov. and description of Blautia wexlerae sp. nov., isolated from human faeces. *Int J Syst Evol Microbiol* 2008;58(8):1896–902. <https://doi.org/10.1099/ijs.0.65208-0>.