

# A Specific Prebiotic Mixture Added to Starting Infant Formula Has Long-Lasting Bifidogenic Effects<sup>1-3</sup>

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## Abstract

There is some evidence that early colonization of the intestine affects the composition of the intestinal microbiota after weaning. In the present study, the effect of prebiotics administered from the first day of life on fecal counts of bifidobacteria and lactobacilli were studied during and after the administration of the prebiotics. In this double-blind, randomized, placebo-controlled, explorative study, 20 newborns of hepatitis C virus-infected mothers who decided not to breast feed due to their concerns regarding their plasma viral load were randomly assigned to either a formula with 8 g/L of a specific prebiotic mixture (short-chain galacto-oligosaccharides and long-chain fructo-oligosaccharides, ratio 9:1) or a formula containing the same amount of maltodextrin (placebo). Clinical examination including anthropometric measurements, microbiological analysis of fecal samples, and blood leukocyte population analysis were performed at birth and 3, 6, and 12 mo age. At the age of 12 mo, hepatitis B vaccine-specific IgG serum titers (Hepatitis B virus surface antibodies) were also measured. Prebiotic supplementation resulted in more fecal bifidobacteria ( $P < 0.0001$ ) and lactobacilli ( $P = 0.0044$ ) compared with the placebo group. These differences between the groups were maintained during the second half of the first year without any prebiotic supplementation. There was no influence of the different diets on anthropometric data or the measured immunological variables. The data from this small explorative study indicate that early colonization of the intestine might have long-lasting effects on the composition of the intestinal microbiota. *J. Nutr.* 141: 1335–1339, 2011.

## Introduction

There is increasing evidence that the intestinal microbiota plays a crucial role in postnatal development of the immune system (1–3). There is broad consensus that breastfeeding is essential and is the best option for optimal development of the intestinal microbiota, resulting in a healthy and balanced immune system (2). Consequently, many attempts have been made to influence the intestinal microbiota composition of bottle-fed infants toward that found during breastfeeding. Most of these experiences exist for prebiotics (4,5) and probiotics (6). More recently, a combination of both, so-called synbiotics (7) or fermented milk (8,9), have also been used as potential treatments to achieve a bifidogenic effect in formula-fed infants.

Oligosaccharides are the main prebiotic factor in human milk (10–13). Because their structure is very complex, they are not yet

available for the production of infant formulas. Thus, research activities have focused on the development of nonhuman milk oligosaccharides as an alternative (14). Several compounds of different structures have been identified and investigated (5).

Since 2002, several infant formulas containing a specific mixture of short-chain galacto-oligosaccharides (scGOS)<sup>7</sup> and long-chain fructo-oligosaccharides (lcFOS) have been on the market (15). Studies in infants with a familial history of atopy/allergy fed such prebiotic formulae demonstrated a reduced risk of atopic dermatitis accompanied by an antiallergic antibody profile in plasma (16,17). Similarly, antiallergic effects have been demonstrated in animals sensitized with ova albumin (18) or cow milk proteins (19,20). In these latter animal studies, it was demonstrated that so-called regulatory T-cells were involved (20), indicating that the inhibition of allergic symptoms were not only due to Th2 damping and/or Th1 stimulation. In addition, a reduction in infectious episodes was observed in infants at risk of developing allergy/atopy (21) as well as in those without such risk (22). In summary, the experimental data (23) as well as the results of studies in humans (5) suggest an immune-modulating effect of this prebiotic mixture (24).

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<sup>3</sup> Supplemental Table 1 is available from the "Online Supporting Material" link in the online posting of the article and from the same link in the online table of contents at [jn.nutrition.org](http://jn.nutrition.org).

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<sup>7</sup> Abbreviations used: HCV, hepatitis C virus; lcFOS, long-chain fructo-oligosaccharide; scGOS, short-chain galacto-oligosaccharide.

For ethical reasons, a certain period of breastfeeding has to be accepted. Because the early imprinting of intestinal microbiota is heavily influenced by early breastfeeding (25), it was of particular interest to obtain data from infants fed the formula from the first day of life. Particular interest was given to the weaning period and the second half of the first year of life to evaluate the stability of the fecal counts of bifidobacteria and lactobacilli after intervention.

In addition, there is still a debate about which biomarkers are really relevant for the evaluation of a normal balanced “healthy” immune system (26). As explorative biomarkers, leukocyte populations and lymphocyte subpopulations, total IgE levels, and hepatitis B virus antibody titers in response to hepatitis B vaccination were examined in this group of infants.

Therefore, this explorative study was initiated to evaluate the influence of a specific prebiotic mixture administered from the first day of life on the development of intestinal microbiota in formula-fed infants with a focus on fecal counts of bifidobacteria and lactobacilli during and after intervention with prebiotics; the above-mentioned explorative immune parameters were also examined.

## Materials and Methods

**Study population and methods.** This randomized, placebo-controlled, double-blinded trial was conducted at the Pediatric Department of the San Paolo Hospital in Milan between January 2002 and December 2005. Infants born from hepatitis C virus (HCV)-infected mothers were eligible for the study. Although it is not the policy of the hospital to recommend formula feeding in these cases, some mothers decided not to breastfeed due to their concerns regarding the relevant plasma HCV viral load before delivery.

The study was approved by the San Paolo Hospital Ethical Committee and written informed consent was obtained from all parents of all enrolled infants.

Inclusion criteria were a gestational age between 37 and 42 wk, a birth weight appropriate for gestational age, and exclusive formula feeding from the first day of life. Prematurity, breastfeeding, major malformations, and HCV infection were exclusion criteria.

Infants who developed metabolic, endocrinologic, and immunologic disorders, lactose intolerance and/or allergy to cow’s milk as well as assumption of other pre- or probiotics were planned to be excluded from the study.

The infants were tested for exclusion of vertical HCV infection at birth and at the age of 1 mo. Two negative results of measurement of qualitative plasma HCV viral load were the basis to exclude vertical HCV infection (27).

The infants were randomly assigned to receive a regular bovine milk formula (Aptamil, Milupa) either supplemented with 8 g/L prebiotics or a similar quantity of maltodextrin as placebo (Milupa) from birth to 6 mo of life. During the second half-year of life, the infants received regular bovine milk-based, follow-on formula without any supplementation.

The prebiotic mixture used in the study consisted of scGOS derived from enzymatic synthesis from lactose and lcFOS separated from chicory inulin in a ratio of 9:1 (15).

The infants underwent clinical examination at birth and 3, 6, and 12 mo of age. During this examination, anthropometric measurements (weight, length, head circumference) using standard methods (28) and a medical examination were performed. Clinical data concerning infections or antibiotic treatments during the month before each medical examination were registered. Fecal samples were obtained for measurements of counts of bifidobacteria and lactobacilli as well as fecal pH.

Additionally, 2 mL venous blood was obtained for routine analysis of leukocyte populations and plasma total IgE titers. At the end of the study, an extra 1 mL blood was obtained for measurement of anti-hepatitis B virus surface specific antibody serum titers (1 mo after the 3rd vaccine dosage was administered).

Fecal counts of bifidobacteria and lactobacilli were measured as previously described (29). Fecal pH was measured by using a Handylab pH meter (Schott Glas) equipped with an Inlab 423 pH electrode (Mettler-Toledo).

Immunophenotyping of peripheral blood lymphocyte T cell subsets was performed by multiparameter flow cytometry on a FACScan flow cytometer (Becton Dickinson).

The IgE titer and specific IgG anti HBs-antigen titer were measured by Electro-Chemi-Luminescence Immuno Assay (ECLIA, Roche Diagnostics).

**Statistics.** The results were analyzed on a per protocol basis. Time-balanced randomization was performed with the software RANCODE (IDV Gauting) with a random permuted block size of 4. Data were compared between the 2 formula groups by 2-sided Mann-Whitney U test. For analysis of the fecal counts of bifidobacteria and lactobacilli as the primary outcomes, 95% CI of the mean were calculated using the Wilson score method for each time point.  $P < 0.05$  (2-tailed) was considered significant. Data are presented as mean  $\pm$  SD or mean (95% CI). The statistical analysis was performed using SAS (SAS Enterprise Guide 4.1) for Windows (SAS Institute).

## Results

Twenty of 22 enrolled infants completed the study. One infant (placebo group) failed due to noncompliance and 1 infant discontinued the follow-up for unknown reasons (prebiotic group).

At enrollment, the 2 groups did not differ in terms of mode of delivery, gestational age, gender, weight, length, or head circumference at birth (Table 1).

All infants had a normal weight gain, length growth, and head circumference increment, and there were no differences between the groups for all measurements (Table 2). None of the infants underwent any antibiotic treatment or suffered from any clinically relevant infection during the month before each evaluation.

The prebiotic supplementation influenced the number of bifidobacteria and lactobacilli in the feces ( $P < 0.005$ ). The counts of bifidobacteria and lactobacilli increased during the first 3 mo in both groups ( $P < 0.0001$ ) and remained stable afterwards ( $P > 0.05$ ). Starting from the 3 mo of age evaluation, the counts of both bifidobacteria and lactobacilli were higher in the prebiotic group ( $P < 0.013$ ). This difference persisted even after discontinuation of the prebiotic supplementation at 7 mo of age (Fig. 1; Table 2).

There was an influence of the supplementation on fecal pH ( $P = 0.0005$ ), with significantly higher pH values in the control group (Table 2). Except for lymphocyte T CD3+ titers at 12 mo of age, which were lower in the control group ( $P = 0.017$ ), the groups did not differ at each evaluation in the measured leukocyte populations (Supplemental Table 1). The total IgE

**TABLE 1** Baseline characteristics of the infants that completed the study<sup>1</sup>

	Prebiotic group	Control group
<i>n</i> (M/F)	10 (5/5)	10 (2/8)
Vaginal delivery, <i>n</i>	10	10
Gestational age, <i>wk</i>	39.1 $\pm$ 0.8	39.1 $\pm$ 1.3
Weight at birth, <i>kg</i>	3.32 $\pm$ 0.7	3.29 $\pm$ 0.34
Length at birth, <i>cm</i>	50.2 $\pm$ 2.7	51.2 $\pm$ 1.9
Head circumference at birth, <i>cm</i>	33.4 $\pm$ 0.9	33.9 $\pm$ 1.0
Bifidobacteria, <i>CFU/g stool</i>	5.5 (4.8–6.3)	5.6 (5.0–6.3)
Lactobacilli, <i>CFU/g stool</i>	4.6 (4.0–5.2)	4.8 (4.2–5.4)

<sup>1</sup> Data are mean  $\pm$  SD or mean (95% CI).

**TABLE 2** Formula consumption, weight, length, head circumference, fecal pH, and fecal counts of bifidobacteria and lactobacilli in infants that received prebiotic or control formula from birth to 6 mo of age<sup>1</sup>

Age and Item	Prebiotic group	Control group	P
<b>3 mo</b>			
Formula, mL/d	767 ± 145	779 ± 71.4	0.87
Weight, kg	6.15 ± 0.81	6.27 ± 0.71	0.82
Length, cm	63.1 ± 2.9	62.1 ± 2.4	0.88
Head circumference, cm	39.6 ± 2.8	40.6 ± 1.3	0.36
Bifidobacteria, CFU/g stool	9.1 (8.7–9.5)	6.4 (5.8–7.0)	0.0014
Lactobacilli, CFU/g stool	6.7 (6.1–7.3)	5.2 (4.6–5.9)	0.0125
Fecal pH	5.24 ± 0.32	6.25 ± 0.51	0.0006
<b>6 mo</b>			
Formula, mL/d	622 ± 223	654 ± 234	0.94
Weight, kg	8.99 ± 0.85	7.97 ± 1.03	0.47
Length, cm	69.0 ± 2.6	69.0 ± 3.1	0.88
Head circumference, cm	43.2 ± 1.0	43.4 ± 1.4	0.59
Bifidobacteria, CFU/g stool	9.4 (8.9–10.0)	7.3 (6.9–7.7)	0.0014
Lactobacilli, CFU/g stool	6.9 (6.3–7.5)	5.5 (5.0–6.0)	0.0054
Fecal pH	5.15 ± 0.39	6.19 ± 0.54	0.0011
<b>12 mo</b>			
Formula, mL/d	310 ± 105	374 ± 156	0.44
Weight, kg	10.9 ± 1.26	9.78 ± 1.21	0.08
Length, cm	78.5 ± 4.2	77.4 ± 5.0	0.42
Head circumference, cm	46.0 ± 1.0	46.9 ± 1.9	0.30
Bifidobacteria, CFU/g stool	8.9 (8.6–9.3)	7.1 (6.8–7.4)	0.0016
Lactobacilli, CFU/g stool	6.7 (6.2–7.2)	5.4 (5.1–5.8)	0.0042
Fecal pH	5.88 ± 0.33	6.20 ± 0.58	0.30

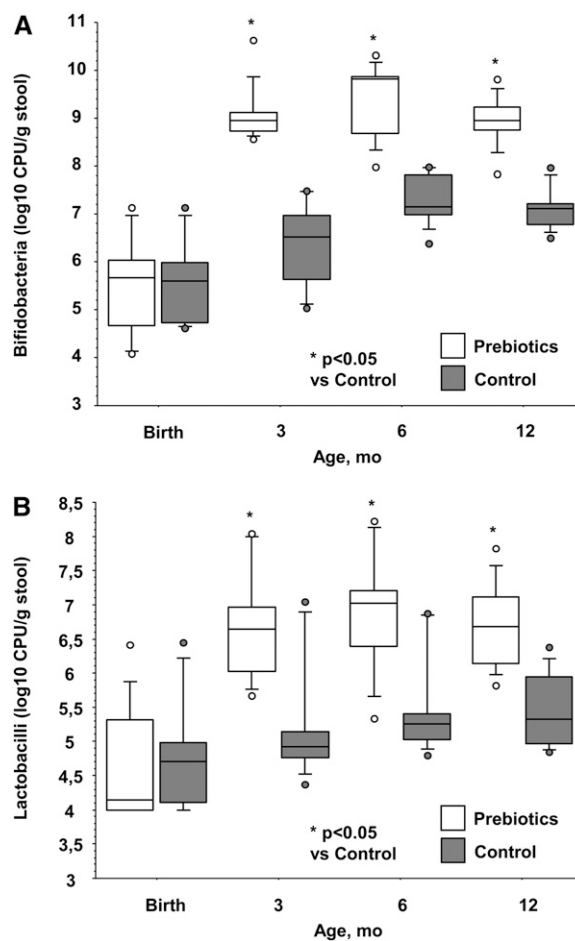
<sup>1</sup> Data are mean ± SD or mean (95% CI).

titers increased from 3 mo of age to the end of the study ( $P = 0.03$ ). The diet did not influence the IgE levels in serum ( $P = 0.27$ ). At 12 mo of age, the anti-hepatitis B virus surface antibody titers did not differ between the feeding groups (data not shown). Both formulas were well tolerated with no adverse effects recorded.

## Discussion

In this study, the prebiotic mixture of scGOS:lcFOS in a ratio of 9:1 significantly increased numbers of fecal bifidobacteria and lactobacilli, as found in several studies (5). The interesting finding in this study is that the early imprinted intestinal microbiota remained relatively stable even after weaning. This is in line with a study using classical microbiological techniques (30) as well as a study performed with molecular-based methods (31). Both studies, as in the present study, demonstrated a substantial stability of bifidobacteria after weaning, confirming data from the early 1980s (32).

Apart from the early imprinting of the intestinal microbiota, the genotype of the host could have an important impact on the stability of the intestinal ecosystem as well (33). The present study did not allow us to conclude whether early imprinting or other factors play a role in the observed stability of the number of fecal bifidobacteria and lactobacilli. The hypothesis that the early colonization might have an effect is supported by the observation that differences in early colonization between infants born by caesarean and vaginal delivery (34) can have long-lasting effects as well (35,36). A similar observation was reported by



**FIGURE 1** Bifidobacteria (A) and lactobacilli (B) counts in fecal samples from infants that received prebiotic or control formula from birth to 6 mo of age. Data are shown in box plots (medians and 95% CI),  $n = 10$ .

Jernberg et al. (37) in infants treated with antibiotics. The impact of the treatment persisted up to 2 y. Furthermore, Roger et al. (38) reported a greater diversity of Bifidobacterium populations in breast-fed than in formula-fed infants, an effect persisting up to 18 mo of age. On the other hand, Rinne et al. (39) studied the effect of early administration of probiotics and could not find any significant interference with composition or quantity of gut microbiota.

In the present study, only culturing methods were used to quantify bifidobacteria and lactobacilli, which is a limitation of the study (40). However, direct comparison of the technique used in the study with the FISH technique demonstrated nearly similar results at a concentration of 8 g prebiotics/L formula (24).

In animal experiments (19), the scGOS:lcFOS mixture has shown immune modulation capacity. These experimental data are in line with the observation that in infants with a familiar history of atopy/allergy, the same prebiotic mixture induced a significant reduction in allergy-related symptoms at 6 mo of age (17). This effect was still seen at an age of 2 y (41), indicating an immune-modulating and -imprinting capacity of the prebiotics used.

In the present study, there was no effect on different leukocyte populations that might indicate that these parameters are not suitable to investigate developmental aspects of the immune system. Raes et al. (42) also found no significant effect of the same prebiotic mixture on basal blood immune variables. However, in

the same cohort, fecal secretory IgA titer was significantly higher in infants fed the supplemented formula (43), indicating an effect of the specific prebiotic mixture on the mucosal immune system.

The vaccine-specific antibody production or T cell function are classified as markers with high suitability in terms of biological relevance, sensitivity, and practical feasibility (26). In the present study, no influence of the prebiotic supplement on hepatitis B vaccination response could be observed. This might be partially related to methodological aspects such as the optimal timing to measure vaccination response during infancy. The vaccine response is very robust. For this reason, subtle effects induced by prebiotic diets might be difficult to detect. If the infants were vaccinated using suboptimal vaccination protocols as is done in animal models (18), it might be possible to demonstrate vaccination improvement. For ethical reasons, suboptimal vaccination in infants is not possible.

More clinical trials are needed to identify predictive biomarkers in human infants that describe the development of the immune system. One example might be Ig-free light chain, which predicts several immune disorders in humans, including infants. Very recent data already indicate that this biomarker can be analyzed easily in plasma from infants and that this biomarker predicts the onset of allergic disorders such as atopic dermatitis, which can significantly be affected by the prebiotic diet. Thus, in future studies, these new biomarkers for immune development should be analyzed (20).

In summary, the data from this small explorative study indicate that early colonization of the intestine might have long-lasting effects on the composition of the intestinal microbiota. This must be confirmed in larger studies additionally using molecular techniques of characterization of intestinal microbiota.

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