Original Article

Effects of probiotic or prebiotic supplemented milk formulas on fecal microbiota composition of infants

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The aim of the study was to evaluate whether supplementation of milk-formulas with prebiotic fructo-oligosaccharides or a probiotic, *Lactobacillus johnsonii* La1 (La1), could modulate the composition of the fecal microbiota of formula-fed infants, compared to breastfed (BF) infants. Ninety infants close to 4 months of age were randomized into one of three groups to be blindly assigned to receive for 13 weeks: a) an infant formula (Control), b) the same formula with fructo-oligosaccharides (Prebio), or c) with La1 (Probio). At the end of this period, all infants received the control formula for 2 additional weeks. Twenty-six infants, breastfed throughout the study, were recruited to form group BF. Fecal samples were obtained upon enrolment and after 7 and 15 weeks. Bacterial populations were assessed with classical culture techniques and fluorescent in situ hybridisation (FISH). Seventy-six infants completed the study. On enrolment, higher counts of *Bifidobacterium* and *Lactobacillus* and lower counts of enterobacteria were observed in BF compared to the formula-fed infants; these differences tended to disappear at weeks 7 and 15. No major differences for *Clostridium, Bacteroide*s or *Enterococcus* were observed between the groups or along the follow up. Probio increased fecal Lactobacillus counts ($P<0.001$); 88% of the infants in this group excreted live La1 in their stools at week 7 but only 17% at week 15. Increased *Bifidobacterium* counts were observed at week 7 in the 3 formula groups, similar to BF infants. These results confirm the presence of higher counts of bifidobacteria and lactobacilli in the microbiota of BF infants compared to formula-fed infants before dietary diversification, and that La1 survives in the infant digestive tract.

Key Words: breastfeeding, milk formula, fructooligosaccharides, infants, intestinal microbiota, Lactobacillus johnsonii La1, prebiotic, probiotic.

Introduction

The colonization of the newborn gastrointestinal tract by bacteria is a complex process which implicates competition for oxygen, nutrients and ecological niches, and is strongly influenced by infant diet, breast or formula feeding. Human milk contains secretory IgA, immune cells, lactoferrin, and lysozyme which hinder pathogen proliferation. It also contains high levels of oligosaccharides (~ 10g/L) which stimulate selectively the growth of some bacterial species, resulting in higher counts of bifidobacteria and lower counts of enterobacteriaceae compared to the microbiota of formula-fed infants.[1,2] Weaning changes the colonic microbiota, decreasing bifidobacteria and lactobacilli and concomitantly increasing *Bacteroides*, *Enterobacter*, *Clostridium*, *Enterococcus*; the resulting microbiota resembles to that of the adult.[1] It is considered that the predominance of bifidobacteria and lactobacilli in the digestive tract generates positive effects upon the host's health and for this reason efforts are being made to modulate the luminal environment with the aim of promoting bacterial populations that more closely imitate those of breastfed infants.[1,3] Theoretically, this may be achieved through the administration of probiotics or prebiotics. Probiotics are microorganisms capable of resisting gastric acidity and bile salts and survive their transit along the gastrointestinal tract of the host where they may regulate the autochtonous microbiota and exert health-promoting functions.[4]

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Lactobacillus johnsonii La1 (La1) is a well-described probiotic strain which has been shown to adhere to intestinal epithelial cells through its lipoteichoic acid, a major cell wall constituent.[5] La1 is capable of modulating local and systemic immunity.[6,7] and antagonizing the sCD14-mediated proinflammatory response induced by LPS in intestinal cell lines.[8] Furthermore, La1 has been shown to exert inhibitory activities against a wide range of pathogens both in adults and children.[9-11]

Prebiotics are non-digestible carbohydrates which are widely used by the food industry. Inulin and fructo-oligosaccharides (FOS) which are extracted from tubers such as chicory or Jerusalem artichoke are among the best described.[12] These molecules cannot be digested by the host and are fermented by the colonic microbiota, selectively stimulating the growth of bifidobacteria.[13]

Regular intake of prebiotics has also been associated with health-promoting effects such as immune stimulation, improved calcium absorption and decreased triglyceridemia and cholesterolemia.[14] In this study, we evaluated the effects of a milk formula supplemented with La1 or FOS upon the colonic microbiota of bottle-fed infants compared to breast-fed infants, using culture methods and fluorescent in-situ hybridization (FISH).

Subjects and methods

Subjects

The study was designed as a prospective, randomized and blinded clinical trial carried out in infants recruited at the "La Faena' Health Care Center in the South Eastern Health District in Santiago, Chile. Parents of potential partici-pants were contacted at the regular Pediatric Clinic of the Health Care Center and, after careful explanation of the scope and aims of the project, those who agreed to the participation of their infants signed a written consent form. The project was approved by the Ethics Committee for Research in Humans of INTA, University of Chile.

Healthy infants born at term, of either sex, 3.5 months old, with birth weight between 3,000 and 4,200 grams, who had not received antibiotic treatments in the month prior to enrolment were included in the study protocol. Exclusion criteria were multiple births, presence of any degree of malnutrition, or gastrointestinal, renal or other chronic diseases.

Study design

The study comprised four diet groups: a) a breastfed group (BF) consisting of infants whose mothers maintained breastfeeding until the end of the observation period (at least 15 weeks after enrolment); b) a control group (Control) who received a standard infant formula available in the local market (Nan 2®, Nestlé Chile, Santiago, Chile); c) a prebiotic group (Prebio) who received the same formula but with FOS at a concentration of 2 grams per liter of prepared formula (Raftilose P95®, Orafti, Tienen, Belgium) and, d) a probiotic group (Probio) who received the same formula but enriched with 10^8 living La1 per gram of powder. The formula groups included infants who had been spontaneously weaned at least 14 days before the beginning of the study; between their weaning and incorporation to the protocol, they were fed a milk formula provided nationwide by the Ministry of Health. Each group received the study diet for 13 weeks following enrolment; in the two weeks following the end of the observation period, all formula-fed infants were given only the standard Nan 2® formula. The sample size was calculated as follows: experience with FISH counts for bifidobacteria gives a SD for log-counts of 0.6. In order to detect a difference in log-count of 0.5 at α=0.5 and a power of 80%, 24 subjects per diet group are required. To compensate for a 15% drop-out, 29 subjects per diet group had to be enrolled, i.e. a total of 116 infants for the four groups. Assignment of the infants to one of the three formula diets was carried out using a computer-generated randomization table. All formula products were letter-coded by the manufacturer such that neither the investigators, the field personnel nor the parents receiving the products were aware of its composition. The code was broken after the data analysis ended. Formulae were placed into cold storage and delivered to the field station at "La Faena" as required, where they were also kept refrigerated. Formulae were periodically tested by Nestlé to ascertain that the probiotic concentrations remained constant during the time course of the study (analysis at release, and 15 and 24 months after production). A total of 3kg of powdered formula were delivered monthly for each child and mothers were carefully instructed on its appropriate preparation. Mothers were also informed that their infant should not receive yogurt or any other fermented foodstuffs throughout the duration of the study. Children who received antibiotics for any condition during the follow up period were excluded from the protocol.

Data and sample collection

Parents were advised to report with their infant every 15 days at the Pediatric Clinic established for this study for health status evaluation and for weight and body length measurements. On this occasion, mothers received a standardized Diet Record Form designed to register prospectively information about food intake for a two week period; items registered were volumes of formula intake, possible intake of yogurt or other fermented food-stuffs, any non dairy products provided to the infants, and possible adverse reactions (including spitting up, vomiting, diarrhea, skin rashes, nasal discharge, cough and difficulty breathing). One week after receiving the Form at the Health Center the child was visited at home by a Registered Nurse from the Project who reviewed in detail with the mother the information accumulated. The nurse took advantage of this visit to stress the importance of the proper preparation of the formula, the need to avoid providing yogurt or similar products and underlined the need for a visit to the Field Station in a week's time. If the mother failed to keep the appointment, the nurse would visit the home and drive the mother with her child to the Station for consultation with the pediatricians participating in the project. In this way, each mother had a contact with the research team at least once weekly. If the infant became ill, mothers had free access to the Project pediatricians. The nurses also undertook unscheduled visits to the homes to verify the compliance with the instructions provided. All children had to take at least 500 ml of the allocated formula daily. The protocol was

inserted within the framework of the National Nutrition Program of the Ministry of Health and in consequence, around weeks 15-16 of the project, when infants were 6 months of age, a vegetable soup and some mashed fruits had to be introduced in the infant's diet to comply with the governmental dispositions. Fecal samples were collected on the day of enrolment (sample 1), after 7 weeks of ingesting the study diets (sample 2), and 2 weeks after the study diet had been interrupted, when all the infants were fed the standard Nan 2® formula (sample 3).

Bacterial cultures

For culture studies, 5 to 10 grams of feces were collected with a sterile spatula immediately after emission, placed into a sterile glass container placed in an anaerobic jar with an Anaero Gen pack, and stored at 4ºC until transport to the laboratory. No more than 2 hours elapsed between sampling and arrival at the laboratory for immediate processing. An aliquot of stool was homogenized, serially diluted, inoculated onto semi-selective media, and incubated under anaerobic conditions. *Bifidobacterium* species were cultured in Eugon Agar, *Lactobacillus* in MRS Agar with antibiotics, *Clostridium* in NN Agar and *Bacteroides* in Schaedler Agar with antibiotics. After incubation, the colonies were counted and further identified. Lactobacilli and bifidobacteria strains were identified by microscopy, and biochemically using the API gallery system (BioMérieux, Paris, France), API 50 CHL gallery for lactobacilli and API ID 32A gallery for bifidobacteria, respectively. *Enterococcus* and *Enterobacteria* were cultured under aerobic conditions in Bile-Azide Esculin Agar and in Violet Red Agar, respectively.[15] Bacterial counts are expressed as $\log_{10}$ colony-forming units (CFUs) per gram of fresh fecal sample, with detection limit at 3.30 cfu/g. Duplicate stool samples obtained on the same occasion were placed into cryotubes containing sterile glycerol, frozen in liquid nitrogen and kept at –70ºC. These samples were shipped periodically to the Nestlé Research Centre (Vers-chez-les-Blanc, Lausanne, Switzerland) for La1 determination and to the University of Reading for fluorescent *in situ* hybridization (FISH) study. Presence of La1 was monitored by random amplification of polymorphisms DNA (RAPD) fingerprint[16,17] using the following oligos GGTTGGGTGAGAATTGCACG and CGGCCAGCTGGTCAGCC (Microsynth, Balgach, Switzerland); this technique identifies strain-specific fingerprints.

For detection of enteropathogens, a rectal swab was obtained on the same occasion as the sample just described and conserved in transport medium. The swab was plated onto culture media for the isolation and identification of *Salmonella* and *Shigella (*XLD Agar with previous inoculation in pre-enrichment and enrichment broths*)*, *Campylobacter* (Skirrow Agar) and *E. coli* classical serotypes, invasive and enterohemorrhagic (EMB and MacConkey Agar). Strains biochemically considered as pathogens were confirmed by using the API gallery system or by PCR and confirmed by serological tests.

Fluorescent in situ hybridisation (FISH)

The probes used in the study were Bif164 and Chis150, specific for *Bifidobacterium* and Clostridium histolyticum cluster including C. perfringens, respectively.[18,19] These were commercially synthesized and 5' labeled with the fluorescent dye Cy3 (MWG Biotech, Germany). The nucleic acid stain DAPI (4', 6-diamidino-2-phenylindole) was used for total counts.[20] Samples were diluted (1:3 v/v) and fixed overnight at 4°C with 4% (w/v) paraformaldehyde; the cells were washed with PBS, resuspended in a mixture of PBS/ethanol (1:1 v/v) and stored at – 20°C.[26] Sixteen microliters of the PBS/ethanol cell suspension were added to 200 µl of pre-warmed hybridization buffer (40mM Tris-HCl, 1.8M NaCl, pH 7.2) and 64µl of HPLC grade water (Fisher Scientific, Loughborough, UK). Ninety micro-liters of the hybridization mixture were then added to 10µl of each probe (50ng/µl) and hybridized for 24h at 50°C. The cells were washed at their respective hybridization temperatures for 30 min in 5 ml of wash buffer (20mM Tris-HCl, 0.9M NaCl, and pH 7.2). The cells were then vacuum filtered onto a 0.2 µm Isopore membrane filter (Millipore Cor-poration, Watford, UK) which was then mounted on a microscope slide with SlowFade (Molecular Probes, Leiden, The Netherlands) and examined using a Nikon Eclipse E400 fluorescent microscope. The 455nm-excitation filter was used to illuminate DAPI stained cells and the 510nm filter was used to count the hybridized cells. At least fifteen random fields of view were counted on each slide and the average count used for analysis. The probes used in this study were Bif164 with a sequence of 5'-CATCCGGCA TTACCACCC-3'[18] and Chis150 with a sequence of 5'-TTATGCGGTATTAATAT(C/T)CCTTT-3'[19].

Statistical analysis

Results were expressed as means ± SD. Data were analyzed as "Intention To Treat" (ITT) taking in account all available data, including those from drop-out subjects, and as "Per Protocol" (PP) including only those infants who completed the study. The number of observed adverse events per subject was compared by one-way ANOVA. Fecal bacterial levels were log-transformed and compared between the three formulas using one way ANOVA. In case of positive ANOVA results, formula groups were compared between them with the Tukey pair-wise multiple comparison test. The breast-fed group was compared to each of the formula group using the two sample t-test.

Results

Characteristics of the study groups

A total of 116 infants were enrolled; because of drop outs, the number of infants under observation decreased, with 76 completing the entire protocol (34.0% drop out rate). Reasons for dropping out were intercurrent illness in 7 subjects (17.5%), antibiotic use in 15 (37.5%), and voluntary withdrawal from the protocol or non-compliance with the diet in 18 (45%). None of the withdrawals was associated with adverse reactions to the formulas. No

significant differences in drop out rates were observed between the four groups.

All formulas were well tolerated by the subjects, and the average formula intake measured during the three days before sample 3 was similar for infants from the 3 groups: 133 ± 35, 126 ± 20, and 138 ± 33 ml/kg of body weight per day for the Control, Prebio and Probio groups, respectively (ITT analysis). This resulted in the daily intake of about $1.8x10^8$ La1 per day for the Probio group. None of the infants showed clinical signs of lactic acidosis during the observation period, and the number of adverse events per infant, including upper and lower respiratory infections and diarrheal episodes was not significantly different between the four groups (one way ANOVA, $P>0.05$; data not shown). As shown in Table 1, no differences between the groups were observed for weight, height, weight for height, weight for age and height for age z-scores (National Center for Health Statistics, NCHS) on the day of enrolment (sample 1) as well as during the study (data not shown). The PP analysis gave similar results as the ITT analysis.

Effects of diets on fecal bacterial counts (PP analysis)

Enumeration of total fecal bacteria by DAPI staining is shown in Table 2. The total numbers of microorganisms were comparable in the different dietary groups although the total bacterial count in the Probio group was moderately higher than that of the Prebio group at sample 1.

Fecal bifidobacteria, as evaluated by culture methods (Table 3), showed no differences between groups, either at samples 2 or 3. Only at sample 1, the BF group had higher bifidobacteria counts than the Probio group. Comparisons probably failed to demonstrate differences due to the high standard deviations. When *Bifidobacterium* concentrations were evaluated by FISH, standard deviations were lower (Fig. 1), and significant changes became apparent: at sample 1, bifidobacteria counts were higher in the BF group than in the 3 formula groups. At sample 2, no differences were observed between the 4 groups and, at sample 3, bifidobacteria counts were higher in the BF group than in the Probio group. Variations were more marked when the *Bifidobacterium* population was expressed as percentages of the total bacterial population, particularly in the Prebio group.

As shown in Table 3, BF infants had significantly lower levels of fecal enterobacteria than the formula groups at samples 1 and 2; however, in the BF infants, fecal enterobacteria significantly increased at sample 3 to reach levels comparable to those in the formula groups. Evaluation of the total *Lactobacillus* population (Table 3) showed higher levels in infants of the BF group on the day of enrolment ($P<0.001$). No changes were observed in the Control or Prebio group throughout the study, while a significant increase was observed in the Probio group at sample 2, such that total Lactobacillus counts

Table 1. Characteristics on day of enrolment (sample 1) of the 4 experimental groups (mean ± SD).

	BF	Control	Probio	Prebio	Significance *
ITT analysis					
N	26	33	25	32	
% females	46	61	48	44	
Weight (kg)	6.74 ± 0.71	6.44 ± 0.70	6.22 ± 0.80	6.58 ± 0.65	NS
Height (cm)	62.04 ± 2.11	61.45 ± 2.11	60.76 ± 2.77	61.95 ± 2.16	NS
NCHS W/H z-score	0.76 ± 0.81	0.61 ± 0.88	0.46 ± 0.87	0.57 ± 0.83	NS
NCHS W/Age z-score	0.83 ± 0.77	0.52 ± 0.78	0.24 ± 0.91	0.60 ± 0.78	NS
NCHS H/Age z-score	0.19 ± 0.89	-0.04 ± 0.73	-0.19 ± 0.72	0.08 ± 0.83	NS
PP analysis					
N	20	23	18	20	
% Drop out	23.1	30.3	28.0	37.5	
Weight (kg)	6.70 ± 0.74	6.39 ± 0.62	6.40 ± 0.83	6.53 ± 0.71	NS
Height (cm)	62.18 ± 2.20	61.30 ± 2.05	60.80 ± 3.00	62.15 ± 2.28	NS
NCHS W/H z-score	0.64 ± 0.83	0.62 ± 0.72	0.70 ± 0.85	0.43 ± 0.66	NS
NCHS W/Age z-score	0.77 ± 0.81	0.54 ± 0.70	0.46 ± 0.92	0.60 ± 0.82	NS
NCHS H/Age z-score	0.24 ± 0.95	-0.03 ± 0.75	-0.12 ± 0.66	0.22 ± 0.89	NS

*One way ANOVA for comparison between the 3 formulas; two sample t-test for comparison between BF and each formula.

Table 2. Evaluation of total bacteria in infant's stools by DAPI staining and direct observation by fluorescence microscopy. (Mean ± SD).

	BF	Control	Probio	Prebio	ANOVA (formula groups) (*P*)
Sample 1	10.78 ± 0.17	10.71 ± 0.27	10.87 ± 0.25*	10.65 ± 0.24	0.039
Sample 2	10.67 ± 0.09	10.72 ± 0.23	10.87 ± 0.18	10.70 ± 0.35	NS
Sample 3	10.62 ± 0.17	10.68 ± 0.24	10.77 ± 0.27	10.61 ± 0.31	NS

* Probio>Prebio

Table 3. Fecal excretion of *Bifidobacterium*, *Lactobacillus*, *Enterobacteria*, *Clostridium perfringens*, *Bacteroides* and *Enterococcus* evaluated by classical culture methods (log_{10} (CFU)/g stool) and fecal excretion of *Clostridium histolyticum* cluster evaluated by FISH (log_{10} (bacteria)/g stool) during the time course of the study in the four experimental groups

	BF	Control	Probio	Prebio
Bifidobacterium				
Sample 1	9.72 ± 1.70[a]	8.23 ± 2.60	7.82 ± 1.87[a]	8.68 ± 1.74
Sample 2	9.49 ± 2.35	9.55 ± 2.33	9.99 ± 1.53	9.41 ± 1.90
Sample 3	9.11 ± 2.54	10.11 ± 1.67	9.12 ± 1.60	9.72 ± 1.97
Lactobacillus				
Sample 1	6.27 ± 1.74[a]	3.92 ± 1.80[a]	3.46 ± 0.75[a]	3.69 ± 1.46[a]
Sample 2	5.13 ± 1.89[b, c]	3.43 ± 0.92[b, d]	5.64 ± 2.38[d]	3.70 ± 1.54[c, d]
Sample 3	4.97 ± 2.34[e]	3.95 ± 1.57	4.17 ± 1.50	3.61 ± 1.01[e]
Enterobacteria				
Sample 1	7.17 ± 2.83[a, b]	8.96 ± 0.93[a]	8.76 ± 1.43	9.18 ± 1.79[b]
Sample 2	7.52 ± 2.43[c]	9.33 ± 1.37[c]	8.79 ± 1.63	9.49 ± 1.32[c]
Sample 3	8.94 ± 1.80	9.62 ± 0.93	8.89 ± 1.15	9.14 ± 1.34
C. perfringens				
Sample 1	3.87 ± 1.35	4.46 ± 1.31	4.25 ± 1.29	4.16 ± 1.27
Sample 2	4.28 ± 1.55	4.80 ± 1.34	4.87 ± 1.54	4.20 ± 1.18
Sample 3	4.37 ± 1.50	4.66 ± 1.25	4.04 ± 1.21	4.22 ± 1.08
C. histolyticum				
Sample 1	8.17 ± 0.27	8.22 ± 0.30	8.14 ± 0.38	8.23 ± 0.41
Sample 2	8.08 ± 0.25	8.07 ± 0.30	8.16 ± 0.42	8.03 ± 0.25
Sample 3	8.14 ± 0.27	8.13 ± 0.34	8.05 ± 0.37	8.07 ± 0.26
Bacteroides				
Sample 1	4.98 ± 1.68	5.64 ± 1.99	5.60 ± 1.97	5.05 ± 1.72
Sample 2	5.10 ± 1.79	5.82 ± 1.82	5.44 ± 2.02	5.02 ± 1.45
Sample 3	5.80 ± 2.22	5.92 ± 1.74	6.09 ± 1.71	5.57 ± 1.75
Enterococcus				
Sample 1	6.30 ± 1.88	6.86 ± 1.57	6.12 ± 0.93	6.54 ± 1.50
Sample 2	6.04 ± 1.72	6.63 ± 1.36	6.72 ± 1.22	7.29 ± 1.23
Sample 3	6.81 ± 1.18	7.10 ± 1.41	6.80 ± 1.53	7.10 ± 1.09

Bifidobacterium: Sample 1: BF>Prebio (P<0.01)[a]
Enterobacteria: Sample 1: BF<Control (P<0.01)[a], Prebio (P<0.02)[b]
Sample 2: BF<Control, Prebio (P<0.01)[c]
Lactobacillus: Sample 1: BF>Control, Probio, Prebio (P<0.001)[a]
Sample 2: BF>Control (P<0.001)[b], Prebio (P <0.02)[c]
Probio> Control, Prebio (P<0.05)[d]
Sample 3: BF>Prebio (P<0.03)[e]
Comparisons refer to the same setter exponents.

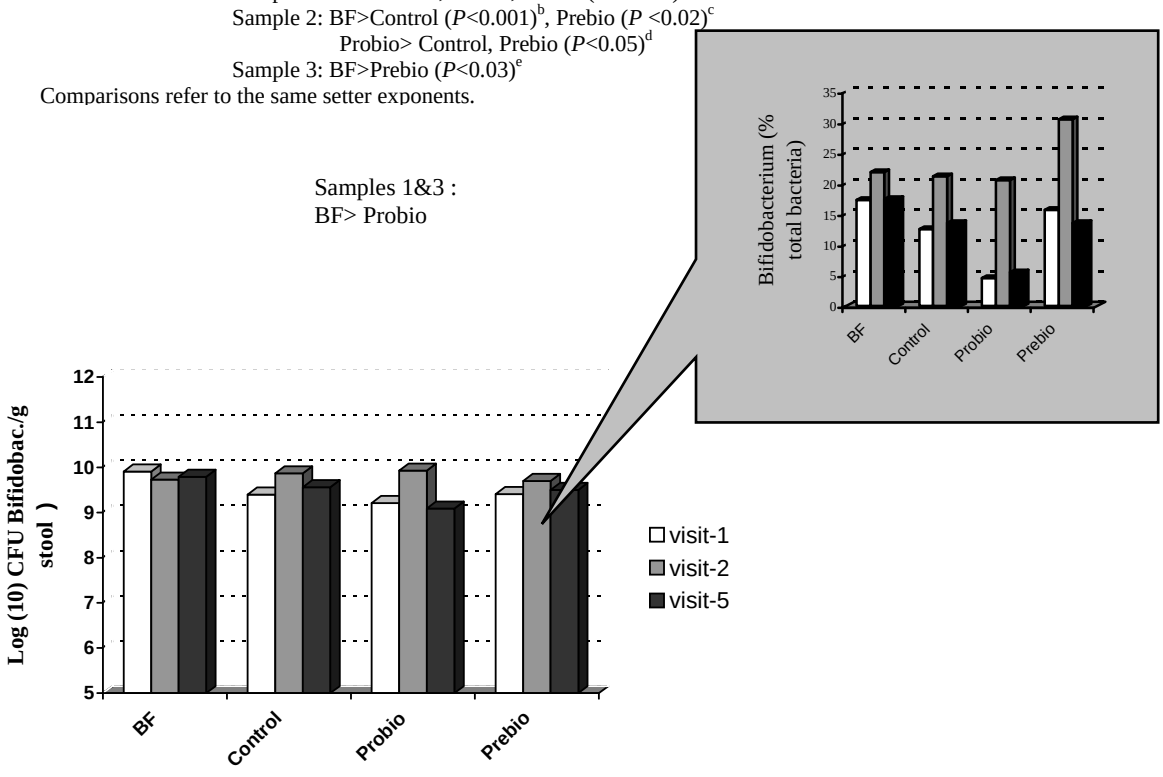

Figure 1. Fecal excretion of *Bifidobacterium* species at samples 1, 2 and 3 in the four study groups, as evaluated by FISH . sample1-t-test: BF> Control (P=0.03), Prebio (P=0.04), Probio (P<0.01); sample3-t-test: BF> Probio (P=0.001). The insert shows *Bifidobacterium* population as a percentage of total bacteria: samples 1&3 – t-test: BF> Probio (P<0.001).

were higher than in the other two formula groups at this time; after interruption of the Probio formula, Lactobacillus tended to return to their pretest levels.

The presence of fecal La1 was also determined in all the infants (Table 4). At sample 1, before the beginning of formula administration, one child was La1-positive in the control and Probio groups. The infant who was La1-positive in the Control group remained positive throughout the study. Fifteen (88%) of the infants who ingested the La1-containing formula excreted the bacteria in their stools on sample 2; however, two weeks after they had interrupted the intake of the formula, only 3 children (17%) continued excreting La1, but with levels similar to those observed at sample 2.

Table 4. Number of La1-positive infants and fecal excretion of La1 in the four experimental groups at the three steps of the study

		BF	Control	Probio	Prebio
Sample 1	Nº of La1-positive subject	0	1/22	1/18	0
	Log CFU La1/g stool	0	5.79	3.30	-
Sample 2	Nº of La1-positive subject	0	1/22	15/17	0
	Log CFU La1/g stool	0	4.86	6.38±1.11	-
Sample 3	Nº of La1-positive subject	0	1/22	3/18	0
	Log CFU La1/g stool	0	6.20	5.21±1.73	-

No differences between groups were observed in fecal *C. perfringens* counts evaluated by culture methods, neither in the counts of *C. histolyticum* cluster detected by FISH (Table 3). No changes in fecal *Bacteroides* or *Enterococcus* (Table 3) populations were observed when comparing the groups in the three stages of the study, nor were intra-group variations observed throughout its time course.

Effects of treatments on fecal enteropathogens

Search of enteropathogens in stool samples from the four groups showed absence of *Salmonella*, *Shigella* or entero-invasive *E. coli* (EHEC). Two major entero-pathogens were isolated in this study, enteropathogenic *E. coli* (EPEC) and *Campylobacter jejuni*, but no differences were found between the groups studied. EPEC were detected in 12%, 9%, 9% and 16% of stools from the BF, Control, Prebio and Probio groups, respectively and *C. jejuni* was detected in 4% and 3% of stools from the BF and Control groups, respectively, and was not detected in the other two groups.

Discussion

The colonic microbiota is a complex ecosystem which plays an important role in the maintenance of health and in the pathogenesis of various diseases.[3] During the last decade, efforts have been made to influence this microbiota through the administration of substances and/or microorganisms such as prebiotics, probiotics or synbiotics with the aim of selectively stimulating the growth of bacteria capable of health-promoting functions, specially bifidobacteria or lactobacilli. In the present study, we examined whether supplementation of milk-formulas with a prebiotic or a probiotic strain modulates the fecal microbiota of bottle-fed infants; these results were compared with the fecal flora of BF infants. Although some studies have observed a predominance of coliforms or *Bacteroides* in the colonic microbiota[21,22], *Bifidobacterium* is usually the predominant genus in BF infants.[23,24] Our results confirm these observations because on the day of enrolment, bifidobacteria accounted for almost 20% of the total fecal population of the BF infants, while percentages as low as 4.6% were observed in the formula-fed infants. Moreover, in the first sampling, fecal counts of lactobacilli, another "beneficial" species, were also higher in the BF group. This may be explained by the presence of galacto-oligosaccharides in the human milk and also by the release in the intestine of bioactive peptides resulting from the digestion of human milk proteins, which are known to stimulate the growth of both *Bifidobacterium* and *Lactobacillus* in the colon.[25,26] Infants fed cow's milk-formulas that do not contain such factors, develop a more complex microbiota with higher counts of facultative anaerobes, *Bacteroides* and clostridia.[21,26-28] Our results at the time of enrolment confirm partially these observations because fecal enterobacteria counts were higher in formula-fed infants, with no differences for *Clostridium*, *Bacteroides* or *Enterococcus*, compared to the BF group.

As previously discussed, *Bifidobacterium* counts at the beginning of the study were higher in BF compared to formula-fed groups, but this difference disappeared at the time of the second fecal sampling, maybe due to the beginning of food diversification at weaning. An increase of bifidobacteria was expected in the Prebio group, as fructo-oligosaccharides are a preferential substrate for these microorganisms.[6] This increase of bifidobacteria in the Prebio group was not significant when expressed as absolute counts, but it became significant when expressed as percentage of the total bacteria population detected by FISH. In the case of Probio the increase of bifidobacteria may be explained as a response to La1 in the intestinal lumen; in effect, it has been recently shown that this microorganism synthesizes and releases fructans that stimulate *Bifidobacterium* growth.[29] We recently observed a similar finding in adult volunteers consuming a La1-containing product.[30]

No differences in lactobacilli counts were observed between the 3 formula-groups. Throughout the study, a decrease in fecal lactobacilli was observed and a similar change was found for *Bifidobacterium* in the BF group; simultaneously with this decrease, a moderate increase in

enterobacteria and *Enterococcus* was observed, suggesting that their colonic microbiota was gradually becoming more complex. As expected, our results showed a significant increase of fecal lactobacilli in the Probio group between the first and the second samples, their counts being higher than in the other formula-fed groups. Addition of La1 to the infant formula was interesting as this probiotic has been shown to stimulate local and systemic immune responses, to modulate mucosal inflammatory processes,[6,7] and to interfere with a wide range of pathogens.[8-11] We observed fecal excretion levels of about $2.4x10^6$ CFU of living La1/g stool in 88% of the infants of the Probio group at the second fecal sampling, reflecting its survival during transit along the gastrointestinal tract. Although it is thought that ingested lactobacilli may compete with the autochthonous microbiota, this was not observed in our study in the Probio group, probably because lactobacilli remained subdominant in the microbiota despite the intake of La1. Two infants from the Probio group did not excrete La1 and as only one stool sample was obtained during the period of formula intake, it is difficult to know whether this absence was due to problems in sample processing or to host factors. Two weeks after completing administration of the formula, La1 was present in the feces of only 3 of the subjects (17%), but at lower fecal concentrations ($1.6x10^5$ CFU/g). This confirms data obtained with other strains of *Lactobacillus*[31] and *Bifidobacterium*[32] indicating that probiotics do not colonize the gastrointestinal tract, but are eliminated after their administration ends. On the other hand, one infant from the control group appeared to be colonized with lactobacilli genetically close to La1; the molecular technique was unable to differentiate between the two bacteria. Similar findings occurred in a clinical trial carried out in Peruvian children with *Lactobacillus* GG.[33]

We cannot compare the culture and FISH techniques for the *Clostridium* because the former detects *C. perfringens*, whereas FISH detects the *C. histolyticum* cluster which includes *C. perfringens* and other *Clostridium* species. Regarding bifidobacteria, again different results were obtained with the two techniques, which confirms Gibson's former observations.[34] However, the standard deviations of results obtained by FISH in our study were lower than those obtained by culture, allowing detection of differences in bifidobacteria counts between groups, which were not observed by culture methods.

Finally, we were also interested in evaluating whether pre or probiotic supplementation decreased pathogens in the intestinal tract of the infants. We previously observed that an acidified milk with *S. thermophilus* and *L. helveticus* decreased asymptomatic fecal shedding of enteropathogens in children[35], a possible cause of chronic environmental enteropathy.[36, 37] In the present study we did not observe effects on the detection of enteropathogens possibly associated with the prebiotic or the probiotic due to their low frequency in the fecal samples. This is probably related to the considerable decrease in microbiological contamination of the environment resulting from improved socioeconomic and hygienic conditions in Chile.

In conclusion, this study confirms a predominance of bifidobacteria and the presence of lactobacilli in higher counts in BF infants than in formula-fed infants. The concentration of fructo-oligosaccharides used in this study showed little effect on the host microbiota, whereas regular intake of a formula containing La1 leads to fecal lactobacilli counts similar to those in BF infants.

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Original Article

Effects of probiotic or prebiotic supplemented milk formulas on fecal microbiota composition of infants

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含有益生菌或益生元的婴儿配方奶的补充对婴儿的排泄物微生物组成的影响

本研究的目的在于评价与母乳喂养（ＢＦ）的婴儿相比，用含益生元的左旋寡糖或者一种益生菌的约氏乳酸菌 La1 (La1)是否能调节配方奶喂食的婴儿排泄物微生物的组成。90 名 4 个月左右大的婴儿随机分成 3 组中的一组任意地接受 13 周：a)一个婴儿配方（对照），b) 婴儿配方加了左旋寡糖的配方，或者 c) 婴儿配方加约氏乳酸菌(Probio)。在 13 周结束后，所有的婴儿吃 2 周对照组配方。在这个课题中，26 个接受母乳喂养的婴儿形成 BF 组贯穿整个课题。粪便样本分别在课题开始前、第 7 周和第 15 周采集。细菌群落数量用传统的培养技术和荧光杂交法测定。76 名婴儿完成了本实验，在课题开始前，与配方喂食组相比、BF 组的婴儿粪便中双歧杆菌和乳酸菌数量比较多，肠杆菌数量比较少，在 7 和 15 周时这些差别渐渐消失了。而梭菌属、似细菌或者肠球菌在这两组间课题开始前或者接下来的实验中均没有差异。Probio 增加了粪便中乳酸菌数量；这组中 88％的婴儿在第 7 周时大便中排出活的 La1，而在第 15 周时只有 17％的婴儿排出。在 3 个配方组中，在第 7 周双歧杆菌数量增加，与 BF 相似。这些结果证明了与配方喂食组婴儿相比，在饮食多样化之前，母乳喂养婴儿菌丛中大量的双歧杆菌属和乳酸菌属的存在，并且在婴儿消化道有存活的 La1。

关键词：母乳喂养、配方奶、左旋低聚糖、婴儿、肠道微生物、约氏乳酸菌 La1、益生元、益生菌。