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Effect of infant formula with probiotics on intestinal microbiota

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SUMMARY. Weaning during infancy refers to the initiation of complementary food to breast milk. During weaning, there are significant changes on the gastrointestinal microbiota. Deleterious alterations of the gastrointestinal microbiota can result in pathological processes while measures that stimulate its development and stability, like the use of probiotics, are beneficial. The mechanisms by which probiotics achieve their effects have not been clearly established. Present work compares the microbial composition of feces from infants that were weaned to regular family food, formula with probiotics (*B. Lactis BL* y *S. Thermophilus*) or formula without probiotics. Accordingly, analysis of rDNA of microbial fecal samples by molecular techniques was used. Formula with or without probiotics was well tolerated and safe for all participating children. Probiotics present in formula were viable and susceptible to culture. There was not difference on physical growth or development among all participants. The microbiota of children supplemented with formula with- or without probiotics was different than that observed in children supplemented with regular food. It was not possible to determine enrichment of *B. Lactis BL* and *S. Thermophilus* in the feces of children that consumed the probiotics. Present work contributes to the understanding of probiotics effects in human health.
Key words: Microbiota, probiotics, infant formula, DGGE, Ecuador.gram, assessment of knowledge, nurses; nursing assistants.

RESUMEN. **Efecto de formula infantil con probioticos en la microbiota intestinal.** Durante el periodo de la alimentación complementaria en la infancia, se producen cambios significativos en la microbiota intestinal. Alteraciones que afecten negativamente a la microbiota pueden resultar en procesos patológicos mientras que medidas tendientes a estimular su desarrollo y estabilidad, como el uso de alimentos probióticos, podrían ser beneficiosas para la salud. Los mecanismos por los cuales los alimentos probióticos logran estos efectos beneficiosos no se han establecido claramente. La presente investigación compara la composición microbiana de las heces de infantes que recibieron como alimentación complementaria: la comida regular de la familia, formula infantil con probióticos (*B. Lactis BL* y *S. Thermophilus*) o formula infantil sin probióticos. Con este propósito se analizaron por métodos moleculares el rDNA microbiano en muestras de heces fecales de los infantes participantes. Las formulas infantiles con o sin probióticos fueron bien toleradas por los infantes. Los probióticos presentes en la formula infantil correspondiente fueron viables y susceptibles de ser cultivados. No hubieron diferencias en el crecimiento físico ni en el desarrollo de los infantes. La microbiota de niños que recibieron formula infantil con o sin probióticos fue diferente de la microbiota de niños complementados con la comida regular. No fue posible evidenciar enriquecimiento de *B. Lactis BL* y *S. Thermophilus* en las heces de niños que consumieron la formula con probióticos. El presente trabajo contribuye a una mejor comprensión de los efectos de los probióticos en la salud humana.
Palabras clave: Microbiota, probióticos, formula infantil, DGGE, Ecuador.

INTRODUCTION

Breast milk is the ideal food for infants during the first six months of life. After this period, complementary feeding is necessary to fulfill infant's nutritional requirements (1). During the period of complementary feeding (weaning) that can last up to two years, there is a gradual replacement of breast milk with family food (1). The introduction of complementary feeding causes significant changes on the gastrointestinal microbiota (2). During weaning there is also an increased incidence of gastrointestinal and respiratory infections with the consequent nutritional deterioration (3).

The gastrointestinal microbiota is a complex ecosystem that has several nutritional and immunological related functions like stimulation of intestinal maturity, maintenance of the mucus barrier, increase in the absorption of nutrients, protection against infectious agents and stimulation of the immune system (4). Conditions that alter the composition of the normal microbiota, for example the use of antibiotics, can provoke pathological processes such as diarrheic syndromes. Conversely, measures that stimulate the development and stability of the normal microbiota, such as the use of pre- or probiotics, have been associated with resistance to infections (5). In this sense, it is important to note that the composition of the intestinal microbiota varies depending on the individual's diet (6).

Probiotics are live microbial feed supplements that are marketed as products that improve the intestinal microbial ecosystem providing beneficial effects to the host (7). Probiotics have proven useful in the prevention of diarrhea in people who travel to developing countries, diarrhea caused by the use of antibiotics, and diarrhea caused by rotavirus (8).

Currently, there is an ample use of probiotics worldwide although scientific data that would support its use is scarce (9). The mechanisms by which probiotics achieve their beneficial effects on human health have not been clearly established. Similarly, there are few studies that have evaluated the changes in the gastrointestinal microbiota during and after the consumption of this type of foods (9). This lack of information is in part due to difficulties in accurately assessing the complex gut microbiota from intestinal content or fecal samples. Normal microbiota includes approximately 400 different species of bacteria, most of which are difficult to culture *in vitro* (2). Traditional methods of bacteria culture can only assess known bacteria. Currently, traditional methods have been complemented with molecular techniques to analyze the microbiota of the gut (10). These approaches study the rRNA and its encoding genes as individual markers for each component of the microbiota (11). Few studies have assessed the effect of probiotics in the composition of intestinal microbiota in general and in particular, there is a paucity of information regarding the use of formula with probiotics in infants during the stage of complementary feeding. Accordingly, the general objective of the present study was to compare the microbial composition of the feces from infants that were weaned to either regular family food, formula with probiotics ((*B. Lactis BL* y *S. Thermophilus*) or formula without probiotics in the city of Quito, Ecuador. In addition, the study evaluated the frequency of acute respiratory and intestinal infections as well as the nutritional status of the infants during the study period.

METHODS

All infants participating in this study were recruited from deprived areas of the city of Quito through a program carried out by Children International Foundation. Exclusively breast fed children between 4 to 6 months of age were randomly divided in three groups as follows, children supplemented with regular family food (n=5; identification number in the study 1, 2, 3, 4, 9); children supplemented with formula with probiotics *Bifidobacterium Lactis BL* and *Streptococcus Thermophilus* (n=5; 5, 6, 13, 14, 15); and children supplemented with formula without probiotics (n=5; 7, 8, 10, 11, 12); All participating children were born at term, weighing more than 2,500 grams, without any acute or chronic illness at the moment of recruitment. Also, children had to be under direct care of an adult. Care givers of children assigned to receive formula, were instructed to prepare the formula supplement following the vendor's recommendations according to the infant's age. The formulas with and without probiotics are currently commercialized in Ecuador. Each formula contains 3.3 grams of protein; 4.4 grams of fat; and 11.8 grams of carbohydrates per 100 Kcal of the product. Both formulas contain casein, vegetable oil, maltodextrin, lactose, vitamins and minerals in accordance with U.S. Food and Drug Administration regulations. The composition of regular family food for those children that did not receive formula was not assessed. Children had a monthly clinical visit where they were evaluated to determine their nutritional status, psychomotor development and the presence of any abnormal pathology. Nutritional status was principally determined by standardized anthropometry.

Monitoring of formula consumption

In order to ensure the adequate intake of the supplemented formula, the following measures were taken. During the monthly medical visit, Dr. GN counted the number of formula cans consumed through that month. Also, children were monitored by weekly phone calls. Caregivers were asked about how they were preparing the formula, compliance of the infants, and general health status of the infants.

Microbiological analysis of formula with probiotics

All infant formulas with and without probiotics used in this study are currently commercialized in Ecuador and were provided by the local vendor. The infant formula with probiotics contains 10^8 colony forming units (CFU) of *B. Lactis BL* and 10^8 CFU *S. Thermophilus* per gram of powder. Infant formulas were stored at room temperature. To test the viability of *Bifidobacterium Lactis BL* and *Streptococcus Thermophilus* in the formula with probiotics, a random sample of the formula with probiotics cans was used. In addition, an analysis of the formula without probiotics was performed as control; formula samples were assayed in triplicate. Initially one gram of the formula was diluted in 10 ml of thyoglycholate broth and cultured aerobically and anaerobically overnight at 37ºC. Subsequently, ten micro liters of broth enriched with bacteria was plated in Raffinose-Bifidobacterium agar, a selective medium for Bifidobacterium (12) and ten micro liters in Streptococcus thermophilus agar, a selective medium for *Streptococcus Thermophilus* (13). Then, single colonies from plates were cultured aerobically and anaerobically in selective medium for *B. Lactis BL* and *S. Thermophilus* (12,13).

To properly identify *B. lactis and S. thermophilus*, morphological as well as molecular methods were used. Conventional Gram staining was done for every culture from the formula with or without probiotics. In addition, PCR analysis using specific primers for *B. Lactis BL* and *S. Thermophilus* were used to further characterize these bacteria in the formulas (14,15). Microbial DNA was isolated from cultures derived from formula with and without probiotics and PCR analysis with specific primers was performed. To determine the specificity of the primers uses, DNA from several pure cultures of different bacteria were used including pure cultures of *B. Lactis BL* and *S. Thermophilus* (16).

Collection of fecal samples

Fecal samples were collected at the beginning of the study, one month and at six months after formula supplementation. All mothers were instructed to place fresh fecal samples immediately at 4°C, and take them to the clinic within 2 hours after defecation, where samples were stored at -20°C until assayed.

DNA isolation from fecal samples

Genomic DNA was extracted using the MoBioä Fecal DNA Sample Kit according to manufacturer recommendations (Carlsbad, CA). With this procedure, 0.25 g of faecal material typically yielded 30 to 50 micrograms of DNA.

Denaturing Gradient Gel Electrophoresis (DGGE)-PCR

The genetic fingerprinting tool, Denaturing Gradient Gel Electrophoresis (DGGE)-PCR was used to differentiate bacterial isolates and identify complexity, dynamics and diversity, including subspecies differentiation of the fecal microbiota (17). Each DNA isolate from the fecal samples was amplified with universal bacteria PCR primers for conserved sequences flanking the variable V3 region of 16s rDNA (534R and 341F), as described previously (18). PCR products were identified by regular agarose gel electrophoresis through the presence of a band of ~ 200 bp (17). To remove single-stranded DNA from the PCR products, 0.75 U of mung-bean nuclease (Stratagene, La Jolla, CA) was added to 15 uL of PCR product (10). After 10 min of incubation at 37ºC, mung-bean nuclease reaction was stopped by the addition of 10 uL of DGGE loading buffer (0.05% bromophenol blue, 0,05% xylene cyanol and 70% glycerol in sterile water). Samples were stored at -20ºC until DGGE analysis. To separate PCR fragments, 35-60% linear DNA-denaturing gradients urea, pormamide, 8% polyacrylamide gels were used (A 100% denaturing solution contains 40% (vol/vol) formamide and 7M urea). Products were separated by electrophoresis at 60°C at 150 V for 2 h, then for 1 h at 200 V. After electrophoresis, gels were silver-stained and scanned using a GS-710 calibrated imaging densitometer (BioRad). When treatment-dependent differences in banding profiles were observed, individual 16S-V3 rDNA bands were excised, re-amplified, cloned and sequenced using an automated sequencing system at the W.M. Keck Center for Comparative and Functional Genomics, (University of Illinois). Sequence data was analyzed using a BLAST (http://www.ncbi. nlm.nih.gov/BLAST/) search for phylogenetic identification.

Statistical analysis

PCR-DGGE band number and the qPCR quantification of each respective bacterial species were subjected to an analysis of variance (ANOVA) using SAS (Version 6.09; The SAS Institute, Cary, NC). The partitioned sources of variation included treatment, day, and their interactions. Specific treatment comparisons was made using Fishers Protected Least Significant Difference test with an assigned P-value of < 0.05. Dendrograms representing clustering patterns of microbial profiles was generated with Diversity Database (Version 2.2.0; BioRad) using Ward's algorithm.

The Ethics Committee of Universidad San Francisco de Quito approved the study.

RESULTS

Subject participants

All children recruited at the beginning of the study completed the trial. General characteristics of children and time of consumption of the formula supplement are indicated in Table 1. The range of days of formula consumption was 180 to 190 days and it was not different among the groups consuming formula. Care providers of all children that received formula with or without probiotics reported that their children had good acceptability of the product and did not report adverse effects that they could associate with formula consumption.

TABLE 1

General characteristics of children and duration of supplementation trial

	No. children (n = 15)	Formula with probiotics (n = 5)	Formula without probiotico (n = 5)	Regular family food (n = 5)
1. Sex M/F	6/9	2/3	1/4	3/2
2. Age at entry (Months and days) +/- SD	7m 10d+/-21d	7m 18d+/-7d	7m 6d+/-25d	7m 6d+/-25d
3. Age at discharge (Months and days) +/- SD	13m 10d+/-21d	13m 17d+/-16d	13m 6d+/-25d	13m 6d+/-25d
4. Days of trial (range)	180 - 190	180	190	182

The follow up anthropometric analysis of participating children is indicated in Table 2. There were not differences in the anthropometric values between the groups at the beginning of the study. There were no differences in physical growth among the three groups of children. The increase in weight, height, and head circumference were similar among the three groups. During the six months of the study it was observed that all children presented slower growth than expected in spite of food availability. However, at the time of recruitment when all children were consuming breast milk, all children had the expected weight, height for their age Table 2.

TABLE 2
Growth data for all participating infants

	Formula with probiotics			Formula without probiotics			Regular food		
	Initial	Final	Gain	initial	Final	Gain	initial	Final	Gain
Weight (Kg)	8.06 +/- 0.51	9.56 +/- 1.06	1.5	7.64 +/- 0.39	9.18 +/- 1.0	1.5	7.72 +/- 0.42	9.42 +/- 0.94	1.7
Height (cm)	68.14	72.24	6.1	65.54	70.68	5.8	65.1	71.0	5.9
Head circumference (cm)	43.52	46.66	3.1	42.94	46.34	3.4	43.4	46.14	2.7

During the monthly clinical visit, care providers for the children were asked for the presence of acute respiratory and intestinal infections. Children in all treatment groups presented similar number of acute infections (data not shown). Due to the limited number of children per treatment group, it is not possible to establish statistical differences among the three groups. The high frequency of acute infections among the three groups could explain the slower growth observed in all the children Table 2.

Microbiological analysis of formula with probiotics

To test the viability of probiotics in the infant formulas, one gram of formula with or without probiotics were cultured as indicated in the methodology section. Positive cultures were obtained only in the samples from the formula with probiotics.

Typical forms of *streptococcus* were observed in cultures selective for *S. Thermophilus* (not shown). Similarly, typical forms of *bacilli* were observed in cultures selective for *B. lactis* (not shown). Further PCR analysis using specific primers for *B. Lactis BL* and *S. Thermophilus* were used to characterize the isolated bacteria from the infant formula with probiotics (14,15). Initially, the specific primers for *B. Lactis BL* and *S. Thermophilus* were used with pure cultures of these bacteria (16), Germany). Pure cultures were used as positive controls for molecular and morphological studies. Isolates from formula with probiotics were subjected to PCR analysis using specific primers for *B. Lactis BL* and *S. Thermophilus*. A unique band that corresponded to the expected size for *B. Lactis BL* or *S. Thermophilus* in the formula isolates as well as in the pure culture controls were observed (not shown). To further test the specificity of the primers for *B. Lactis BL* and *S. Thermophilus*, microbial DNA was isolated from several pure cultures of different bacteria and were subjected to PCR analysis. As shown in Figure 1, only DNA from cultures of *B. Lactis BL* and *S. Thermophilus* amplified a band corresponding to each of these bacteria using the specific primers. Together, this data indicates that bacteria isolated from formula with probiotics corresponded to *B. Lactis BL* and *S. Thermophilus*.

Microbial composition of the feces by Denaturing Gradient Gel Electrophoresis (DGGE)-PCR

To compare the microbial composition of the feces from infants supplemented with either regular family food, formula with probiotics (*B. Lactis BL* y *S. Thermophilus*); or formula without probiotics, PCR-DGGE analysis was carried out as indicated in the methodology section. Fecal samples of participating children were collected before formula supplementation and one and six months after the introduction of the formulas.

The universal primers used in an initial amplification yielded a product of approximately 200 base pairs (data not shown). To separate the PCR products, denaturing 8% polyacrylamide gels were prepared so that 35-60% linear DNA-denaturing gradient was formed (100% denaturant is

equivalent to 7 mol/L urea and 40% deionized formamide). PCR-DGGE was run at 150 V for 2 h at 60°C, then for 1 h at 200 V. As reference markers, ladders representing known bacterial strains were also run to standardize band migration among different gels. After wards, the electrophoresis gels were silver stained and scanned in a GS-710 Calibrated Imagining Densitometer (Gibco). Figures 2 shows typical band patters observed after DGGE analysis. Each gel contained the samples of 5 children and every child had three fecal samples taken before one and six months after complementary food was started. Children 1, 2, 3, 4, and 9 were supplemented with regular family food; children 5, 6, 13, 14, and 15 were supplemented with formula with probiotics; and children 7, 8, 10, 11, and 12 received formula without probiotics.

FIGURE 1
PCR products of 16sDNA V3 region from bacteria isolated from formula with probiotics

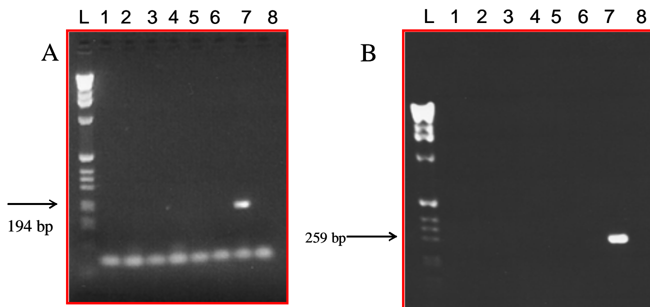
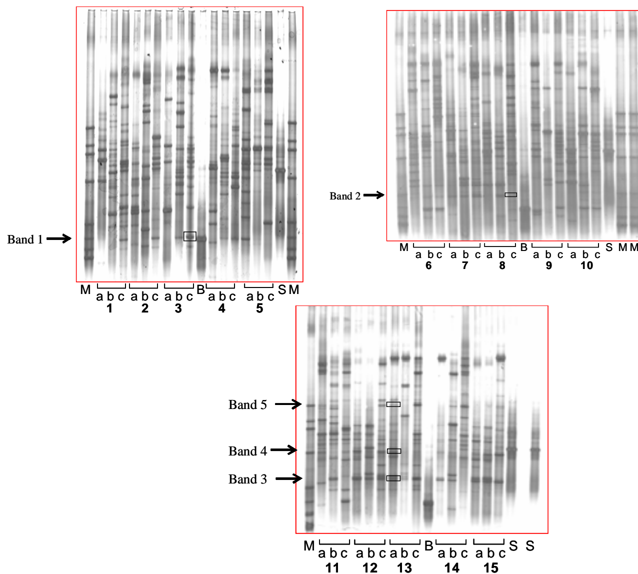


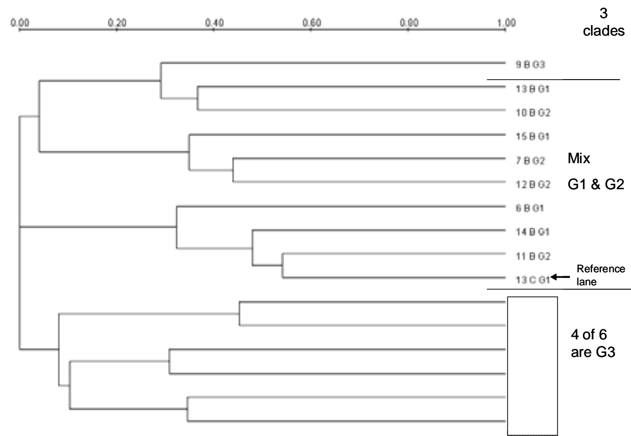
FIGURE 2
DGGE analysis of fecal samples from children supplemented with or without probiotics



The effects of the diet supplement on the number of PCR-DGGE bands expressed in each sample were compared. The number of bands in the samples collected at the beginning of the study was similar among the three groups (not shown). There were not significant differences in the number of bands in the samples taken at one month and six months after the beginning of the study.

The effects of weaning diets on microbial composition were also assessed by cluster analysis based on Ward's algorithm. Ward's algorithm is used to form hierarchical groups of mutually exclusive subsets. This analysis is regularly used to cluster the PCR-DGGE banding pattern based in their similarities and differences (18). The microbial populations of children that received formula or formula with probiotics more closely resembled each other than they did the children who were supplemented with regular food (data not shown). This was more evident after one month of supplementation, children supplemented with regular family food clustered together apart from children that received formula (Figure 3).

FIGURE 3
Dendrogram representing dietary correlations of PCR-DGGE banding patterns in fecal samples from children supplemented with formula with probiotics or without probiotics and regular family food



Analysis of band patterns present in the three treatment groups indicated the presence of common bands in all groups as well as some bands that were predominantly in one or two of the groups (Table 3). To identify the bands, arbitrary numbers were assigned to each band. Band number one was present predominantly in the feces of children supplemented with regular family food group while bands 2 and 3 were more common in the feces of children supplemented with formula with or without probiotics (Table 3). Also, bands 4 and 5 were present in all the groups. Table 3 summarizes the characterization of this selected group of bands that were excised from the DGGE gels for cloning and sequencing as

indicated in materials and methods. Sequence data was analyzed using a BLAST (http://www.ncbi. nlm.nih.gov/ BLAST/) search for phylogenetic identification. These data indicate that supplementation of infants with regular food or formula with or without probiotics can influence the microbial composition of the fecal microbiota.

TABLE 3
Identification of bands excised from PCR-DGGE gels determined by sequence alignment

Band Number	GeneBank accession No	Percent similarity	Identity	Tratment Group
1 (G3)	AY856700	99%	*Bifidobacterium sp.* h12	Regular family food
2 (G1, G2)	AY736853	83	*Bifidobacterium longum bv. Infantis*	Formula with or without probiotics
2 (G1, G2)	AY986349	99%	Uncultured bacterium	Formula with or without probiotics
3 (G1, G2)	AF371550	94%	Uncultured bacterium	Formula with or without probiotics
4 (G1-3)	AY806191	99%	uncultured *Streptococcus* sp.	All treatment groups
5 (G1-3)	AY985751	98%	uncultured Streptococcus sp.	All treatment groups

(G1) = Formula with probiotics, children identification number 5, 6, 13, 14, 15
(G2) = Formula without probiotics, children identification number 7, 8, 10, 11, 12
(G3) = Regular family food, children identification number 1, 2, 3, 4, 9

DISCUSSION

The data presented here indicate that formula with or without probiotics was well tolerated and safe for all participating children. *B. Lactis BL* and *S. Thermophilus* present in the formula with probiotics were viable and susceptible to culture. There was not difference on physical growth or development among children who were supplemented with formula, formula with probiotics, or regular family food. However, all participating children in the trial presented inadequate growth during the six months of the trial in spite of food availability. Inadequate growth could be the result of the frequent acute intestinal and respiratory infections observed in all participants.

Previous work has demonstrated the beneficial effects of formulas containing *B. Lactis BL* and *S. Thermophilus* (19, 20). Corrêa NFO, et al. demonstrated that consumption of *B. Lactis BL* and *S. Thermophilus* was associated with a prevention of antibiotic-associated diarrhea (19). In that study the microbiota composition of participating infants was not assessed. In a recent report Saavedra et al. evaluated the safety and tolerance of a formula with the same probiotics used in the present study in 118 infants demonstrating the safety in their use (20). In that report, children that consumed formula with *B. Lactis BL* and *S. Thermophilus* had lower use of antibiotics and lower reports on intestinal pain than infants that were supplemented with formula without probiotics (20). Similar to the previous report, Saavedra's study did not analyze possible effects of probiotics on microbiota composition of the studied population (20). In the present study, all participating children presented similar number of acute diarrhea and acute respiratory infections. In addition, also contrasting with Saavedra's report, all children had inadequate growth. The differences in growth and the frequency of acute infections in the present study and the ones reported by Saavedra could be due to settings where the studies were carried out. Present study was carried out with a group of mestizo infants (mix of Indians and white Spanish) living in poor neighborhoods in Quito – Ecuador, a developing country whereas Saavedra's study was carried out in the metropolitan area of Baltimore – United States. Other studies carried out in developing countries with probiotics proved useful in developed countries have failed to demonstrate the beneficial effects of these known probiotics (21). For instance, a randomized controlled trial that studied the effect of Lactobacillus strain GG (LGG) in 124 male patients between 1–24 months of age with different severity of diarrhea demonstrated that there was not significant reduction in diarrhea duration in subjects given LGG compared with controls (22). Present report highlights the importance of evaluating a product like a formula with probiotics in different environments such as a developed and developing countries

where ethnicity, sanitation, education, availability of resources (better socioeconomic conditions) are different.

The present data indicate that the microbiota of children supplemented with formula with- or without probiotics was different than that observed in children supplemented with regular food. With the molecular methods used here it was not possible to determine enrichment of *B. Lactis BL* and *S. Thermophilus* in the feces of children that consumed the probiotics. DGGE analysis can only detect microbes that constitute at least one percent of the population in a microbial echo system. It is possible that the consumption of the formula with probiotics at the present doses does not permit an increment of the bacteria enough to be detected by DGGE. Although a dose-effect relationship has been suggested, there are limited studies of pharmacokinetic on probiotics (21). The high frequency of acute diarrhea among the participating children could also modify the microbiota and in that way hindering any effect of the probiotics. Further studies with higher doses of *B. Lactis BL* and *S. Thermophilus* may be necessary to change the microbial composition of feces. Also more research with a greater number of subjects from different geographical areas is needed to correlate clinical data and composition of the microbiota.

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