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Galacto-oligosaccharides Are Bifidogenic and Safe at Weaning: A Double-blind Randomized Multicenter Study

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ABSTRACT

Objectives: The primary objective of this study was to determine the bifidogenic effect of galacto-oligosaccharides (GOS) in a follow-on formula and the effects on other intestinal bacteria. Secondary objectives were the effects on stool characteristics, growth, and general well-being.

Participants and Methods: In a multicenter, double-blind study, 159 healthy infants, formula-fed at enrollment (at 4–6 months), were randomized to an experimental follow-on formula supplemented with 5 g/L (GOS) (77 infants), or to a standard follow-on formula (control, 82 infants). Infants were evaluated at enrollment (study day 1 = sd1), after 6 weeks (study day 2 = sd2), and after an additional 12 weeks (study day 3 = sd3). At each study day, a fresh stool sample for the bacterial counts was collected, and the growth parameters were measured. At sd2, urinary specimens were collected for the evaluation of urinary osmolarity.

Results: At sd2 and sd3, the GOS group had a higher median number (colony-forming units per gram of stool) of bifidobacteria than did the control group (sd2 GOS 9.2×10^9 vs control 4.4×10^9, $P = 0.012$); (sd3 GOS 7.2×10^9 vs control 2.4×10^9, $P = 0.027$). Other bacteria did not show any significant differences between the 2 groups at all study days. The GOS produced softer stools but had no effect on stool frequency. The urinary osmolarity (mOsm/L) at sd2 was comparable in both groups. Supplementation had no influence on the incidence of gastrointestinal side effects or on the growth of the infants.

Conclusions: These data indicate that the addition of GOS (5 g/L) to a follow-on formula positively influences the bifidobacteria flora and the stool consistency in infants during the supplementation period at weaning. No local or systemic side effects were recorded. ***JPGN 48:82–88, 2009.***

Key Words: Follow-on formula—Microflora—Prebiotic—Bifidobacteria—Safety—Stool—Weaning.

The human distal intestinal tract harbors a complex microbial community, which is still insufficiently characterized and which plays a fundamental role in health and disease (1). The intestinal ecosystem provides nourishment, regulates epithelial development, and affects immunity and nonspecific defense resistance of the host and thus modulates health and well-being (1,2).

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The defensive properties of human milk are based on a unique mixture of protective agents and components that modulate the gut microbial ecosystem of the newborn (3,4). The stools of breast-fed infants are dominated by bifidobacteria and, to a lesser degree, by lactobacilli (3). Evidence is accumulating that the presence of a large community of bifidobacteria and lactobacilli, which may account for as much as 90% of the total flora of breast-fed infants, is fundamental for the well-being of the infant (5). A natural approach to modulate the composition of the gut flora is to supply these potentially helpful organisms with selective nutrients to give them a distinctive competitive advantage over other bacteria (5). Of the available food ingredients, dietary carbohydrates that have escaped digestion in the upper gastrointestinal tract

(nondigestible carbohydrates [NDCHs]), but that are fermented in the lower portions of the gut, represent the natural substrates. They are the only food components for which direct evidence of a direct positive effect on the growth of lactic acid bacteria and on health has been reported (6). Although the bifidogenic activity of human milk may be based on a complex set of interactive substances and not on a single component, a sound hypothesis is that the prevalence of bfidobacteria in the stools of breast-fed infants is strongly related to the presence of GOS (5).

During the past few years, experimental evidence has confirmed that NDCHs are well tolerated and have the potential to increase the number of bifidobacteria in the colon (7). Several ingredients have been tested and selected to reproduce the bifidogenic effect of human milk oligosaccharides. A mixture containing 90% short-chain GOS and 10% long-chain fructo-oligosaccharides has been tested in more than 400 infants in several clinical trials (5,8). A strong stimulating effect on the number of bifidobacteria has been clearly documented (5). In some of these trials, an increase in the number of bifidobacteria has been accompanied by a reduction of clinically relevant pathogens in the stools (7,9). The possibility that other oligosaccharides, alone or in different blends and quantities, can be equally effective or more effective in increasing the number of bifidobacteria and in decreasing the number of clostridia, must be seriously considered. The properties of GOS, which have been tested in few animal experiments and in clinical studies in healthy adults and in healthy formula-fed infants, indicate that these substances are the natural candidates to be added to infant formulas, to modulate the gut ecosystem (10–14).

Further research is also needed to allow adequate recommendations for the intake of dietary fiber during weaning and, in general, during the first year of life (15). There are concerns that excessive intake may lead to slow growth, mineral imbalance, and increased fecal energy and water losses in infants fed formula supplemented with NDCHs, but only limited data are available (16). Weaning is a crucial time for the establishment of good eating habits and for the consolidation of favorable colonic microflora, which is also essential to control the rising incidence of allergies (15,16). During this period, new complex carbohydrate foods are included in the diet, and the resident flora must cope with the increase of starch intake, which can itself become an additional source of fermentable material in a gut where not all digestive functions are well developed (17). In addition, commercial weaning foods have different fiber content and carbohydrates, such as fructose from fruit juices that may escape digestion and become colonic foods themselves (17–19). The scarcity of clinical data and the lack of clear and well-founded guidelines for the use of prebiotics during infancy, especially in the weaning period, are matters of serious concern.

Therefore, the objective of this study was to determine in healthy infants the bifidogenic effect of the addition of GOS to follow-on infant formulas. The aim of the study was also to assess the growth of the infants and the possible gastrointestinal side effects associated with the consumption of NDCHs during weaning.

PATIENTS AND METHODS

This was a randomized, double-blind, placebo-controlled, parallel-group study conducted simultaneously at 4 university centers: Ferrara and Turin (Italy) and Las Palmas and Seville (Spain), from June 2001 to May 2005. The ethical committees of all of the centers approved the protocol. Written informed consent was obtained from the parents or legal guardians of all of the participants.

Participants

In this study, 172 healthy infants 4 to 6 months old, whose mothers were not able to provide any breast milk at that time and who met the following inclusion criteria, were considered for eligibility: birth weight $\geq$1500 g, appropriate for gestational age, and exclusive formula feeding with a standard infant formula at the moment of enrollment. Infants were excluded if they met even only 1 of the following criteria: major congenital anomalies, congenital viral infections, family history of allergy (any allergic disease in first-degree relatives), consumption of breast milk, previous treatment with probiotic preparations or feeding with prebiotic or probiotic supplemented formulas, any antibiotic treatment from 14 days before the enrollment until the end of the study period (sd3), and previous introduction of any solid food. Withdrawal criteria were as follows: introduction of solid foods before sd1, assumption of any probiotic products or other prebiotics, any antibiotic treatment, and decision of parents.

Design

Each infant received the same standard infant formula for at least 14 days (pre–study period) before being randomly assigned to receive 1 of 2 follow-on formulas for the subsequent 18 weeks: a standard follow-on formula with 5 g/L maltodextrins (control) or a standard follow-on formula supplemented with 5 g/L GOS (Friesland Foods Domo, Zwolle, the Netherlands; Table 1). A simple randomization procedure was carried out at each center, using a standard pseudo-random number generator with CryptGenRandom (Microsoft). The feeding assignment was determined by the opening of a sealed envelope just before enrollment, and the infants were assigned to a progressive identification code. The powdered formula cans were anonymously labeled with different colored stamps.

Parents were given a diary to record daily milk volumes (at least 230 mL/day, corresponding to at least 1.15 g GOS in the GOS group) and solid foods introduced. The introduction of beikost was accomplished by the parents; the attending pediatrician gave general recommendations and rules, with the indication to completely avoid any food or preparation containing

TABLE 1. *Composition of the follow-on formulas* (per liter)*

	Control	GOS
Energy, kcal	760	760
Lactose, g	58	58
Maltodextrins, g	10	5
Starch, g	23	23
GOS, g	0	5
Fat, g	36	36
Protein, g	17	17
Whey/casein ratio	51/49	51/49
Minerals, g	6.2	6.2

* Both formulas contained vitamins (A, D_3, K, thiamin, B_2, riboflavin, pyridoxine, B_{12}, niacin, pantothenic acid, folic acid, biotin, C), choline, inositol, taurine, and minerals (calcium, phosphorus, magnesium, iron, zinc, manganese, copper, iodine, sodium, potassium, chloride) as recommended by European Directive 91/321/EEC of Commission 14/05/1991 and European Directive 92/52/EEC of the Council 18/06/1992.

prebiotic or probiotics, including yogurt. A second section of the diary concerned the characteristics of the stools: stool frequency and consistency. For each stool, mothers had to express an evaluation of the consistency on the basis of an iconographic score (1 = hard, 2 = formed, 3 = seedy, 4 = soft, 5 = watery). Regurgitation, vomiting, flatulence, and other clinical problems could be recorded in a specific section of the diary.

Infants were evaluated at randomization (sd1), 6 weeks later (sd2), and finally 18 weeks after sd1 (sd3). At each study day, a complete clinical examination and an anthropometric evaluation were performed. Body weight was measured with an electronic baby scale (Clinical Baby Scale Model 727, Seca GmbH, Hamburg, Germany) with an accuracy of ±5 g. The crown-to-heel length was measured with the Harpenden infantometer (Holtain Ltd, Crymcych, UK) with an accuracy of ±1 mm. To search for potential impairment of water and electrolyte balance at sd2, a urine specimen for osmolarity determination was collected from all of the infants enrolled at 1 of the Italian centers (Turin; Osmostat OM620, Daiichi Kagaku, Kyoto, Japan).

Microbiological Analysis

Three stool specimens for microbiological analysis were obtained from each infant in the hospital, directly from the diaper or by rectal stimulation: at randomization (sd1), after 6 weeks (sd2), and 18 weeks after the intervention was begun (sd3). Immediately after stool collection, an aliquot of 0.5 g was homogenized in 4.5 mL of a cryoprotective glycerol transport medium (according to Crowther) (20) and immediately frozen at −80°C for storage (21). The frozen samples were transported in special containers on dry ice to the Institute of Microbiology of Herborn, Germany. All of the samples were thawed under anaerobic conditions at room temperature and introduced into a dilution series whereby decimal dilutions were made up to 9 in a Ringer solution diluent. For the quantitative bacterial determinations, the resulting dilutions were applied to the specific agar plates by use of a spiral plater (Don Whitley WASP2, Meintrup DWS Laboratory Equipment GmbH, Lähden-Holte, Germany) (22). The specific agar plates used were as follows: DP agar (Heipha, No. 20580e) for bifidobacteria, Rogosa agar (Heipha, No. 20680e) for lactobacilli, anaerobic agar (Heipha, No. 3840e) for clostridia, Schaedler GV agar (Heipha, No. 3181e) for *Bacteroides*, and U3G2 agar (Heipha, No. 1030e) for Enterobacteriaceae. After microaerophilic incubation for 2 days at 37°C, the single colonies were counted and identified on the basis of their morphology and biochemical reactions as previously described (23). Colony counts were obtained and expressed as a log of the colony-forming units per gram of fresh feces.

Statistical Methods

Anthropometric data of infants and data from stool characteristics (stool frequency and consistency) showed a normal distribution and are expressed as mean ± standard deviation (SD). In addition, z scores were calculated by use of the northwestern Italy charts (24) for birth data and the WHO growth charts for subsequent measurements (25). Comparability of the control and GOS group was examined on the basis of anthropometric data at birth by use of an independent Student t test. For statistical analysis of anthropometric data during the study, a multifactor analysis of variance (age and formula feeding as factors) was used, followed by Scheffe post-hoc analysis.

The average stool frequency was analyzed during the first 6 weeks of dietary intervention (from sd1 to sd2, 42 days) and calculated by dividing the total number of the stools by 42 days. Stool consistency was expressed by assigning a score to each specimen (1 = hard, 2 = formed, 3 = soft/seedy, 4 = loose/mushy, 5 = watery). The average stool consistency from sd1 to sd2 was obtained by calculating the value of the average consistency of all of the stool specimens and by dividing the resulting value for the total number of the stools of the period.

Data of fecal bacteria counts were not distributed normally and are expressed as medians with the 25% Q–75% Q (interquartile range, IQR). The course of the mean $\log_{10}$ of fecal bacteria counts at week 0 (sd1), week 6 (sd2), and week 18 (sd3) was presented as a curve; areas under the curve of the GOS and control group for the bacteria groups were compared separately by use of unpaired Mann-Whitney tests by ranks. Bacteria counts of the GOS and control group were compared at sd2 and sd3 by multifactor analysis of variance with week and formula as the factors, followed by unpaired Mann-Whitney tests by ranks. A value of $P \leq 0.05$ was regarded as statistically significant. All of the statistical analyses were performed with software package Statgraphics Plus, version 4.1 (Manugistics, Rockville, MD).

RESULTS

Of the 172 infants enrolled in the study, 159 (75 boys, 84 girls) were randomized to the GOS formula (77 infants) or to the control formula (82 infants; Fig. 1). Of these 159 infants, different subgroups of infants were used for analysis of fecal bacteria counts, stool characteristics, and urinary osmolarity.

Neither weights nor lengths of infants differed between the groups or subgroups at birth (Table 2) or at sd1, sd2, or sd3, both corrected and uncorrected for age of preterm

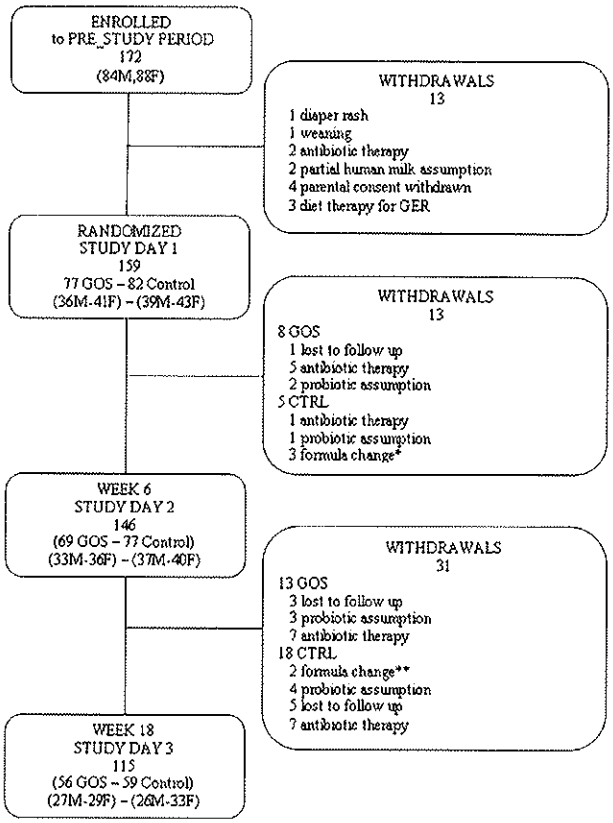

FIG. 1. Flow of participants through each stage of the study. *1 gastroesophageal reflux, 1 enteritis, 1 constipation; **constipation.

infants (15 in the GOS group, 13 in the control group; Table 2).

Fecal bacterial counts (Table 3), analyzed from the 115 infants who completed the 18-week study (56 GOS vs 59 control), were not different between the groups before dietary intervention was started (sd1). In particular, the number of fecal bifidobacteria, expressed as colony-forming units per gram of feces (median IQR), did not differ between groups at sd1 (8.0×10^8) $(1.6 \times 10^8 - 4.0 \times 10^9)$ (GOS) vs (8.7×10^8) $(2.1 \times 10^8 - 4.8 \times 10^9)$ (control) ($P = 0.448$). However, 6 and 18 weeks after dietary intervention was started (at sd2 and sd3), the GOS group had a significantly higher content of fecal bifidobacteria compared with the control group, as follows: sd2 (9.2×10^9) $(1.6 \times 10^9 - 3.4 \times 10^{10})$ (GOS) vs (4.4×10^9) $(6.6 \times 10^8 - 9.2 \times 10^9)$ (control), $P = 0.012$; sd3 (7.2×10^9) $(9.9 \times 10^8 - 1.5 \times 10^{10})$ (GOS) vs (2.4×10^9) $(2.2 \times 10^8 - 7.9 \times 10^9)$ (control, $P = 0.027$). Figure 2 shows the medians and the IQR of $\log_{10}$ (fecal bifidobacteria counts) of both groups at weeks 0, 6, and 18, and the corresponding areas under the curve. The fecal numbers of lactobacilli, *Bacteroides*, clostridia, and Enterobacteriaceae were not significantly different between groups, neither at sd2 nor at sd3. However, the number of lactobacilli tended to be higher in the GOS group at sd3 (4.2×10^4) $(1.0 \times 10^2 - 3.2 \times 10^6)$ (GOS) vs (1.0×10^4) $(1.0 \times 10^2 - 1.1 \times 10^5)$ (control, $P = 0.164$).

Mean stool frequency analyzed in a subgroup of 88 infants, all recruited at the 2 Italian centers (41 GOS vs 47 control), was not significantly different between the groups from sd1 to sd2 (mean daily output [±SD]: 1.68 ± 0.56 GOS vs 1.62 ± 0.54 control, $P = 0.621$). The range of stool frequency was between 0 and 4, without diarrheal episodes in both groups. However, the average stool consistency was significantly influenced by the GOS supplementation, with softer stools, as shown by a significantly higher mean score (arbitrary units, AU; ±SD) in the GOS group (2.78 ± 0.45 GOS vs 2.25 ± 0.58 control, $P < 0.001$). None of the infants fed the supplemented follow-on formula had watery stools on average (maximum score 4.2) at any time during the observation period.

Urinary osmolarity (milliosmoles per liter), analyzed from a subgroup of 52 infants, all recruited in Turin (22 GOS vs 30 control) at sd2, was comparable between both groups: median IQR: 336 (206–485) GOS vs 271 (152–661) control, $P = 0.663$). Also, the incidence of crying, regurgitation, vomiting, and flatulence was not different between the groups (data not shown).

DISCUSSION

Despite a growing literature supporting a variety of possible health benefits of NDCHs, clinical studies performed to evaluate the effects of prebiotics at weaning are relatively few. One double-blind randomized trial has analyzed bifidobacteria in fecal samples of infants randomized to receive weaning foods enriched with a mixture of GOS and fructo-oligosaccharides or weaning products without this mixture; the feeding regimen offered a daily dose of 4.5 g of GOS/fructo-oligosaccharides (26). This study was limited to 20 infants and did not include any information on the growth of the infants enrolled in the trial. The results indicated that enrichment of solid foods with the mixture of prebiotics increased the proportion of bifidobacteria in the stools. Information on other biota was not available (26). Other studies have used only oligofructose and have usually been limited to observations on tolerance and the effects on gastrointestinal function, growth, general health status, and immune response after vaccination but have not investigated the primary effect of prebiotics (ie, impact on the intestinal ecosystem) (27–30). Also, a large, randomized, community-based trial has concluded that cereals supplemented with oligofructose are not associated with any change in diarrhea prevalence or response to *Haemophilus influenzae* type B immunization in children 6 to 12

TABLE 2. *Anthropometric data of study population at birth and during the study*

	No. infants control/GOS	Control (mean ± SD)	GOS (mean ± SD)	*P*
GA, wk	59/56	37.7 ± 3.10	37.7 ± 2.86	0.923
Weight, g	59/56	2950 ± 661	2875 ± 690	0.549
z Score		0.02 ± 1.08	−0.12 ± 0.91	0.612
Length, cm	59/56	48.6 ± 3.2	48.1 ± 3.1	0.467
z Score		0.31 ± 1.09	−0.03 ± 1.20	0.139
Study day 1 (wk 0)				
CA, wk	59/56	18.6 ± 3.5[*]	17.8 ± 4.3[*]	
Weight, g	59/56	6993 ± 717[*]	6969 ± 988[*]	
z Scores		0.20 ± 0.88[*]	−0.12 ± 0.91[*]	
Length, cm	59/56	63.7 ± 2.8[*]	63.3 ± 3.2[*]	
z Scores		0.05 ± 1.19[*]	−0.03 ± 1.20[*]	
Study day 2 (wk 6)				
CA, wk	59/56	24.6 ± 3.4[†]	23.7 ± 4.9[†]	
Weight, g	59/56	7752 ± 760[†]	7725 ± 1042[†]	
z Score		0.33 ± 0.89[†]	0.37 ± 1.09[†]	
Length, cm	59/56	66.8 ± 2.6[†]	66.6 ± 3.1[†]	
z Score		0.28 ± 1.17[†]	0.29 ± 1.16[†]	
Study day 3 (wk 18)				
CA, wk	59/55	36.7 ± 3.6[‡]	35.3 ± 6.7[‡]	
Weight, g	58/55	8878 ± 846[‡]	8821 ± 1198[‡]	
z Score		0.47 ± 0.91[‡]	0.45 ± 1.16[‡]	
Length, cm	58/55	71.4 ± 2.6[‡]	71.3 ± 3.6[‡]	
z Score		0.31 ± 1.14[‡]	0.42 ± 1.33[‡]	

Data are expressed as mean ± SD. Superscripts within rows ([*],[†],[‡]) indicate significant differences between control group and GOS group (multifactor analysis of variance [day and formula feeding] according to Scheffe post hoc analysis). CA = corrected age from term; GA = gestational age.

months old, living in a community afflicted by a high burden of gastrointestinal and other infections (31). Also, in that study the effects of prebiotics on the intestinal ecosystem were not analyzed.

Our double-blind, randomized, multicenter research is, as far as we know, the first clinical study to investigate the effect of low doses of GOS on the number of bifidobacteria and other relevant microbiota in the feces of healthy infants at weaning and on growth, gastrointestinal tolerance, stool consistency, and water balance. The results indicate that GOS, consumed for 18 weeks in a follow-on formula, at a concentration of 5 g/L, together with other weaning foods, stimulates the growth of bifidobacteria throughout the supplementation period but has limited or no impact on other biota. This bifidogenic effect was obtained by use of a single prebiotic agent and at a dosage that was much lower than the concentration of 8 g/L considered safe by the Scientific Committee on Food of the European Community and used in a variety of infant formulas (32). The dietary treatment with low doses of GOS was well tolerated by all of the infants enrolled in the study. In particular, we could exclude any negative impact on water and electrolyte balance, which may have been expected in case of excessive water losses with the stools in infants consuming GOS (32). These reassuring data are different from those recently obtained by Ziegler et al (33), who observed a greater incidence of diarrhea and irritability in term infants fed for their first 4 months of life with a formula supplemented with a prebiotic blend that contained, besides GOS, polydextrose and lactulose (33). By contrast, several other studies have documented the high gastrointestinal tolerability of GOS, alone or in association with fructo-oligosaccharides (7,13). Furthermore, as already reported in other studies, no undesirable effect on weight and length was observed during the entire study period in the infants fed the follow-on formula enriched with GOS. This observation is important with regard to reports of growth delay in children receiving excessive dietary fiber intake (15). The concern that dietary fiber may negatively affect growth by reducing energy supply is not confirmed by our data.

In conclusion, the present study demonstrates that the addition of a relatively small quantity of GOS to an infant follow-on formula is well tolerated by healthy infants at weaning and has no adverse effect on growth. During the supplementation period, GOS can increase the number of bifidobacteria in the feces of infants who are also consuming solid foods. This may represent a safe and practical approach to the modulation of the intestinal ecosystem. Furthermore, GOS supplementation induced soft stools without documented signs of dehydration.

The bifidogenic effect of the supplementation of an infant formula with GOS could contribute to a favorable intestinal microflora also in infants receiving a mixed diet, consuming miscellaneous food products containing many complex chemical entities. Within the limitations of the present study, it can be concluded that feeding a follow-on formula with GOS 5 g/L is bifidogenic and safe

TABLE 3. *Fecal bacterial counts in study population*

	Bifidobacteria	*Bacteroides*	Lactobacilli	Clostridia	Enterobacteriacae	*Escherichia coli*
Study day 1 (week 0)						
GOS group (n = 56)	8.90 (8.20–9.60)	5.91 (4.00–8.79)	4.13 (2.00–6.52)	4.50 (3.30–5.70)	8.46 (7.87–8.87)	8.30 (7.76–8.63)
Control group (n = 59)	8.94 (8.32–9.68)	5.64 (4.00–8.53)	4.43 (2.00–6.67)	4.35 (3.40–5.64)	8.48 (7.87–8.89)	8.32 (7.64–8.69)
P	0.448	0.841	0.735	0.717	0.987	0.924
Study day 2 (week 6)						
GOS group (n = 56)	9.96 (9.21–10.53)	6.96 (4.00–9.07)	4.86 (2.00–7.04)	4.92 (4.08–6.28)	8.69 (8.13–9.07)	8.54 (7.99–8.92)
Control group (n = 59)	9.64 (8.82–9.96)	7.92 (5.93–9.20)	4.44 (2.00–6.00)	5.00 (3.95–5.95)	8.84 (8.47–9.17)	8.57 (8.27–8.96)
P	0.012	0.337	0.367	0.946	0.169	0.333
Study day 3 (week 18)						
GOS group (n = 56)	9.86 (8.99–10.18)	7.95 (6.64–9.60)	4.62 (2.00–6.50)	4.30 (3.00–5.28)	8.65 (8.12–9.13)	8.50 (7.90–8.99)
Control group (n = 59)	9.38 (8.35–9.90)	8.16 (6.30–9.04)	4.00 (2.00–5.05)	4.29 (2.48–5.43)	8.53 (7.96–9.01)	8.33 (7.59–8.83)
P	0.027	0.705	0.164	0.934.	0.204	0.270
AUC weeks 0–18						
GOS group (n = 56)	10.38 (1.04–25.16)	14.07 (−0.31 to 27.73)	0.00 (−26.55 to 23.30)	4.56 (−19.20 to 24.78)	1.15 (−4.08 to 11.64)	2.75 (−5.45 to 13.55)
Control group (n = 59)	0.08 (−6.82 to 17.38)	12.73 (−1.45 to 36.59)	−3.23 (−22.97 to 11.87)	2.99 (−13.55 to 20.90)	2.21 (−6.64 to 13.91)	2.33 (−5.44 to 14.79)
P	0.010	0.731	0.250	0.987	0.756	0.895

Data are expressed as medians [$\log_{10}$ colony-forming units] (25%Q–75%Q). AUC = areas under the curve, expressed as medians (25%Q–75%Q) of arbitrary units.

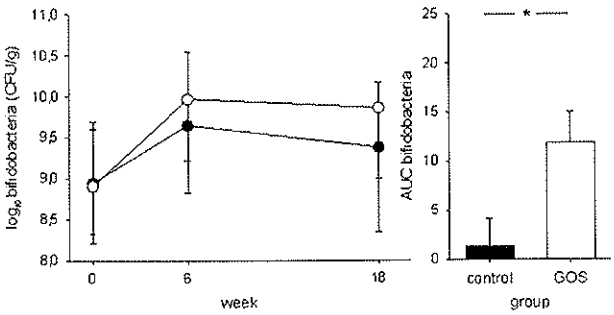

FIG. 2. Median and 25%Q to 75%Q of $\log_{10}$ fecal bifidobacterial counts at weeks 0, 6, and 18, and the corresponding areas under the curve (arbitrary units) of both study groups. Black bars and dots = control individuals. White bars and dots = GOS group. AUC = area under the curve. *Significant difference between GOS and control group ($P < 0.05$; unpaired Mann-Whitney tests by ranks).

during the feeding period. Further studies are required to evaluate other relevant clinical benefits and, above all, the optimal range of effective dosages of GOS in follow-on formulas.

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