Effects of galactooligosaccharide and long-chain fructooligosaccharide supplementation during pregnancy on maternal and neonatal microbiota and immunity—a randomized, double-blind, placebo-controlled study[1–3]

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ABSTRACT

Background: Galactooligosaccharides (GOS) and long-chain fructooligosaccharides (lcFOS) proliferate bifidobacteria in infant gut microbiota. However, it is not known how GOS and FOS influence the microbiota of pregnant women and whether a potential prebiotic effect is transferred to the offspring.

Objectives: We aimed to test how supplementation with GOS and lcFOS (GOS/lcFOS) in the last trimester of pregnancy affects maternal and neonatal gut microbiota. Variables of fetal immunity were assessed as a secondary outcome.

Design: In a randomized, double-blind, placebo-controlled pilot study, 48 pregnant women were supplemented 3 times/d with 3 g GOS/lcFOS (at a ratio of 9:1) or maltodextrin (placebo) from week 25 of gestation until delivery. Percentages of bifidobacteria and lactobacilli within total bacterial counts were detected by fluorescent in situ hybridization and quantitative polymerase chain reaction in maternal and neonatal (days 5, 20, and ≈182) stool samples. Variables of fetal immunity were assessed in cord blood by using flow cytometry and cytokine multiplex-array analysis.

Results: The proportions of bifidobacteria in the maternal gut were significantly higher in the supplemented group than in the placebo group (21.0% and 12.4%, respectively; $P = 0.026$); the proportion of lactobacilli did not differ between the groups. In neonates, bifidobacteria and lactobacilli percentages, diversity and similarity indexes, and fetal immune parameters did not differ significantly between the 2 groups. Mother-neonate similarity indexes of bifidobacteria decreased over time.

Conclusions: GOS/lcFOS supplementation has a bifidogenic effect on maternal gut microbiota that is not transferred to neonates. The increased maternal bifidobacteria did not affect fetal immunity as measured by a comprehensive examination of cord blood immunity variables. *Am J Clin Nutr* 2007;86:1426–37.

KEY WORDS Galactooligosaccharides, fructooligosaccharides, prebiotics, pregnancy, microbiota, fetal immunity

INTRODUCTION

The commensal gut microbiota plays important roles in human health by contributing to host nutrition and by providing a natural defense mechanism against invading pathogenic bacteria (1). The colonization of the neonatal gut starts immediately after birth (2) and is influenced by the mode of delivery (3, 4), the composition of the maternal microbiota (5), and the mode of feeding—ie, breast milk or infant formula (6, 7). In early infancy, the intestinal microbiota further influences the development of the immune system (8); it has been found to be associated with the later appearance of allergies (9–13). Therefore, several strategies were developed to alter the composition of the neonatal gut microbiota (14). Infant formulae were supplemented with either live probiotic bacteria (15), mostly bifidobacteria and lactobacilli (16, 17), or prebiotics (18), such as nondigestible oligosaccharides (19–21), which serve as a selective substrate for the intestinal microbiota. Although numerous potential health benefits were associated with consumption of prebiotics and probiotics (22, 23), the Committee on Nutrition of the European Society for Pediatric Gastroenterology, Hepatology and Nutrition recommended further research to test the efficacy and safety of prebiotics and probiotics in dietetic products for infants (24, 25).

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An alternative approach is the supplementation of pregnant women with prebiotics or probiotics (16), which produces changes in the maternal gut microbiota that may then be transferred to the infant at delivery. We hypothesized that the supplementation with both galactooligosaccharides (GOS) and long-chain fructooligosaccharides (lcFOS) during pregnancy promotes a bifidobacteria-enriched microbiota in the maternal gut and that these bifidobacteria would be transferred from the mother to the neonate. Furthermore, we wanted to know whether this GOS/lcFOS supplementation could affect the development of the neonatal immune system and directly or indirectly influence the neonatal gut colonization process and whether any such effect would be analogous to published effects of probiotics (16, 26) and prebiotics (27). To test this hypothesis, a double-blind, randomized placebo-controlled, prospective pilot-study was designed to investigate the effect of long-term supplementation of pregnant women with GOS/lcFOS on the numbers of bifidobacteria and lactobacilli in the maternal and neonatal gut. Special attention was paid to comprehensive analyses of cord blood (CB) immune parameters.

SUBJECTS AND METHODS

Participants

Healthy pregnant women aged 18–45 y with uncomplicated pregnancies who were scheduled for vaginal delivery at the Department of Obstetrics of the Ludwig-Maximilians-University and the Hospital III Orden (Munich, Germany) were enrolled in the study. Women were included if they planned to deliver in one of the study hospitals, did not intend to donate CB stem cells and were not beyond week 24 of gestation at enrollment. Exclusion criteria were acute or chronic illness, chronic inflammatory bowel disease (eg Crohn's disease or ulcerative colitis), gestational diabetes, intake of antibiotics and antiinflammatory drugs (eg, cortisone, acetylsalicylic acid, or nonsteroidal antiinflammatory drugs), and regular consumption of commercially available, prebiotic- or probiotic-supplemented yogurt. In addition, participants were excluded if they discontinued supplementation for >2 d more than twice during the study.

Compliance was assessed by standardized telephone calls every 3 wk and by a questionnaire at delivery asking how often supplementation was discontinued for >2 d (never, once, twice, or more than twice). Neonates were excluded for obvious malformation, perinatal asphyxia (5-min APGAR score < 7.0 or CB pH < 7.2), or clinical or laboratory signs of a neonatal or maternal infection at delivery. Recruitment began in March 2004 and continued until July 2005, and follow-up ended in January 2006. The Consolidated Standards of Reporting Trials (CONSORT) guidelines (recommendations for improving the quality of reports of parallel-group randomized trials) were followed (28).

Written informed consent was obtained from all participants. The study protocol was approved by the Ethics Committee of the Bavarian College of Physicians.

Study design

The study was performed as a double-blind, placebo-controlled pilot trial. After randomization, participants received sachets with 6 g of a powdered water-soluble product containing either 3 g prebiotics (45.45% short-chain GOS and 4.91% lcFOS with 14.39% glucose, 15.15% lactose, 19.34% maltodextrin, and 0.76% galactose) or 6 g maltodextrin (placebo) to be dissolved in 200 mL of water or milk. The 2 supplements were coded "N" or "O" and were indistinguishable in color, smell, and taste. The number of supplement sachets needed for a 15-wk supplementation period was delivered to the homes of participating women, who were instructed to consume 3 sachets/d from week 25 of gestation until delivery. Detailed instructions were given on the label of each sachet. The women were also asked to maintain their habitual diet throughout the intervention period. Both supplements were manufactured by Numico Research (Friedrichsdorf, Germany); the company kept the codes unbroken, and the database was locked until all samples were analyzed.

Outcome measurements

The primary maternal outcome variables were the relative percentages of bifidobacteria and lactobacilli within the gut microbiota before supplementation and shortly before the expected delivery. Secondary outcomes were stool frequency and consistency, vaginal pH values, acceptance and tolerance of the supplement, and potential side effects (eg, regurgitation or bloating).

The primary neonatal outcome variables were the relative numbers of bifidobacteria and lactobacilli within the neonatal gut microbiota on days 5 and 20 and after 6 mo. The secondary outcome was a comprehensive phenotypical and functional analysis of CB to assess possible effects of GOS/lcFOS via altered maternal microbiota on the neonatal immune system. When >30% of data was missing, that subject was excluded from further study and subsequent statistical data evaluation.

Data collection

At study entry, the atopic status of the family (mother, father, and siblings) was assessed in a standardized face-to-face interview that included information on allergic diseases (eg, hay fever, atopic dermatitis, or allergic asthma diagnosed by a physician) and smoking habits (ie, the number of cigarettes/wk). Subject-completed weekly questionnaires were used to collect data on stool frequency per day, bowel behavior (eg, complaints about abdominal pain, distension, and flatulence), stool consistency, and regurgitation (recorded on a 5-point scale for severity). Women were further instructed to measure their vaginal pH weekly by using CarePlan VpH test gloves (pH scale: 4.0–7.0; Inverness Medical Unipath, Cologne, Germany). Participants were contacted by telephone at 3-wk intervals to promote compliance and to assess the occurrence of side effects and complaints in a standardized manner. At the end of the study, overall tolerance, compliance, and acceptance and the occurrence, intensity, and duration of complaints or side effects were assessed in each subject. Protocol adherence was checked by counting the number of returned sachets at the end of the study. At delivery, neonatal anthropometric measures (ie, weight, length, head circumference, and APGAR score) and maternal birth records were documented. The follow-up questionnaire was adapted from the German Infant Nutrition Intervention study (29, 30) and used to monitor feeding habits, medication, infections and atopic eczema.

Fecal sample collection and microbial analyses

Two maternal stool samples were taken from each subject before supplementation and shortly before expected delivery. Two neonatal stool samples were taken from each infant on days

5 and 20 and at the age of 6 mo (≈day 182). The mothers were asked to deep-freeze the samples within 20 min after collection. The samples were transported in a frozen state and stored at −20 °C until analyses were conducted.

The stool pH was measured directly in thawed samples with the use of a Handylab pH meter (Schott Glas, Mainz, Germany) equipped with an Inlab 423 pH electrode (Mettler-Toledo, Columbus, OH). Fecal samples were diluted 1-in-10 in phosphate-buffered saline and homogenized with the use of a stomacher (IUL Instruments, Barcelona, Spain). The suspensions used for DNA extractions were subsequently frozen at −20 °C until further analyses were conducted.

For the fluorescent in situ hybridization (FISH) analysis, 1 mL of homogenized fecal suspension was fixed in 3 mL freshly prepared 4% (wt:vol) paraformaldehyde in phosphate-buffered saline and incubated overnight at 4 °C. The total number of bacteria was determined by 4'-6-diamidino-2-phenyl indole staining (DAPI; Sigma-Aldrich, Zwijndrecht, Netherlands), and the percentages of bifidobacteria and lactobacilli were determined by using FISH, as described previously (7, 31).

Before DNA extraction, the homogenized fecal samples were thawed at room temperature. DNA isolations were carried out with the NucliSense Isolation Kit (BioMerieux, Boxtel, Netherlands). For the relative quantification of the genera *Bifidobacterium* and *Lactobacillus* and the different species of bifidobacteria and lactobacilli, duplex 5' nuclease quantitative polymerase chain reaction (qPCR) assays were used (20, 21). Briefly, with different primer and probe combinations, a temperature profile consisting of 2 min at 50 °C and 10 min at 95 °C, followed by 45 cycles of 15 s at 95 °C and 1 min at 60 °C min was run on an ABI Prism 7900HT sequence detection system (Applied Biosystems, Nieuwerkerk a/d Ijssel, Netherlands). Thereafter, the percentages were determined according to Liu et al (32). The minimum detection threshold of FISH was 10^6 cells per g wet weight of feces, and the detection limit of the qPCR analyses was 0.00001%.

Blood sampling

Maternal peripheral blood was collected at delivery by venous puncture into 7.5-mL tubes containing EDTA (Vacutainer; BD Diagnostics, Franklin Lakes, NJ). Placental venous CB was collected into 7.5-mL EDTA-containing tubes and 7.5-mL lithium-heparin –containing tubes immediately after delivery and analyzed within 4 h after collection.

Flow cytometry

Whole CB samples (50 μL) were stained with appropriately diluted fluorescein isothiocyante-, phycoerythrin-, phycoerythrin-cyanin 5-, or allophycocyanin-labeled monoclonal antibodies to human CD4, CD8, CD45RA, CD45RO, CD25, CD69, CCR4, and CCR5 (all: BD Biosciences, Heidelberg, Germany); to CCR1, CCR2, CCR6, CCR7, CCR8, CCR9, CXCR3, CXCR4, and CXCR5 (all: R&D Systems, Wiesbaden-Nordenstadt, Germany); and to CRTH2 (Milteny Biotech, Bergisch-Gladbach, Germany) and with appropriate isotype controls (Beckman Coulter, Krefeld, Germany). After being stained, contaminating erythrocytes were lysed with the use of fluorescence-activated cell-sorting (FACS) lysing solution (BD Biosciences). A minimum of 10 000 lymphocytes was acquired (FACS-Canto flow cytometer; BD Biosciences) and analyzed by using FACS-DIVA software (version 5; BD Biosciences). Thresholds were set with forward scatter (FSC) on the x axis and with side scatter (SSC) on the y axis to exclude debris and dead cells. Because CB contains nucleated red blood cells, which overlap with FSC and SSC characteristics of the lymphocytes, a gate was set on $CD4^+$ cells in a $CD4^+$-SSC plot to exclude nucleated red blood cells from analyses. After defining a cutoff according to the isotype controls, we analyzed the percentages of CKR^+- and $CKR^+CD45RA^+$-expressing lymphocytes within the $CD4^+$ T helper (Th) population and the percentages of CKR^+-expressing cells within the $CD4^+CD45RA^+$ population. The same analysis was performed in the $CD8^+$ and $CD8^+CD45RA^+$ T cell populations. For CXCR4, the mean fluorescence intensity was determined. $CD4^+CD25^{high}$-expressing T regulatory cells were measured according to the method of Lundgren et al (33). Only $CD4^+$ cells expressing CD25 with intensities higher than those of the $CD8^+$ cells were included in the analyses. The percentages of $CD25^{high}$ and $CD45RA^+CD25^{high}$ cells were measured in the $CD4^+$ population.

Whole-cord blood stimulation assay

Heparinized CB was diluted 1-in-5 in RPMI-1640 (Gibco Invitrogen Life Technologies, Grand Island, NY). Aliquots of 1 mL diluted CB were stimulated with concavalin A (50 μg/mL; Sigma Chemical Co, Poole, United Kingdom), β-lactoglobulin (100 μg/mL; Sigma), ovalbumin (100 μg/mL; Sigma), lipopolysaccharide (0.1 μg/mL LPS; Sigma); staphylococcal enterotoxin B (0.1 μg/mL; Sigma), *Dermatophagoides pteronyssinus* (10 μg/mL, affinity-purified; Indoor Biotechnologies, Cardiff, United Kingdom), or medium alone in a 24-well culture plate. Cells were incubated at 37 °C in a 5% CO_2− atmosphere for 24 or 48 h, and supernatants were stored at −80 °C until cytokine analyses were performed.

Multiplex array

Cytokines were quantified in culture supernatants with the use of a human multiplex, particle-based, flow cytometric assay (Bio-Plex Suspension Array System; BioRad Laboratories, Hercules, CA) according to the manufacturer's instructions. Data were analyzed by using BIOPLEX-MANAGER software (version 3.0; BioRad Laboratories) with 5 parameter-logistic-regression-algorithms curve fits. Detection limits for the cytokines were 2–32 000 pg/mL. Tumor necrosis factor-α, interferon-γ, interleukin (IL)-1β, IL-2, IL-4, IL-10, granulocyte-macrophage–colony-stimulating factor, and granulocyte colony-stimulating factor concentrations in the supernatants were directly measured without further dilution. Supernatants were diluted 1-in-10 in RPMI to measure IL-6, IL-8, monocyte chemoattractant protein-1 and macrophage-inhibitory protein-1β. Values from negative controls were subtracted, and concentrations were normalized to the number of lymphocytes (for IL-2) or total leukocytes (for all other cytokines) obtained from whole blood counts ($pg \cdot mL^{-1} \cdot 10^3\ cells^{-1}$).

Sample size calculations

The sample size was based on published concentrations of bifidobacteria in fecal samples (7). With a sample size of 15, it is possible to detect a mean difference of 30% in bifidobacteria,

with a probability of 80% and a (2-sided) significance level of 0.05.

Fifteen participants per group were necessary. A computerized randomization list using a 4-block design (total sample size: 30; number of treatments: 2) was used to implement the random allocation sequence. The randomization was generated by Numico Research. At study entry, participating women were allocated by sequential numbers to 1 of the 2 supplementation groups. Participants who failed to complete the study or dropped out were replaced according to the randomization list. We assumed that the study withdrawal rate would be ≥25%. On that basis, we estimated that ≥19 participants should be enrolled into each group.

Statistical analyses

Statistical analyses were performed with SPSS software (version 12.0; SPSS Inc, Chicago, IL). Continuous data were tested by using the Kolmogorov-Smirnov test to determine whether they were normally distributed. Normally distributed data were analyzed by using the 2-tailed *t* test. For nonparametric data, the 2-tailed Mann-Whitney *U* test was used. Descriptive results are expressed as median values with ranges. Intragroup comparisons were performed with the paired Wilcoxon's signed-rank test. For ordinal and nominal (categorical) data, the percentages of bacteria were calculated, and the differences were determined by using Pearson's chi-square test. Differences within one group were calculated by using the McNemar test. In maternal stool samples, the percentages of bifidobacteria or lactobacilli after supplementation were analyzed by using a univariate analysis of covariance with supplementation as the factor and percentages of maternal bifidobacteria or lactobacilli before treatment (baseline) as the covariate. In infant stool samples, the percentages of bifidobacteria or lactobacilli were analyzed by using an analysis of covariance with treatment as the between-subject factor and time as the within-subject factor. Percentages of maternal bifidobacteria or lactobacilli after treatment were included as the covariate. Diversity indexes (DIs; *see* below) were analyzed in the same way.

The percentages of subjects who were positive for specific (single) bacterial species were analyzed by using a general log linear model with the percentage of subjects who were positive for a specific bacterial species, the supplementation, and the time as factors in the model. The bacterial DI was calculated by using the following equation:

$$\mathrm{DI} = (a/b) \times 100 \qquad (1)$$

where a represents the number of found species in a sample, and b represents the total number of analyzed species ($n = 9$ bifidobacteria and $n = 8$ lactobacilli). The bacterial similarity index (SI) in mother-neonate pairs was calculated by using the following equation:

$$\mathrm{SI} = (c/d) \times 100 \qquad (2)$$

where c represents the number of mother-neonate species that matched (ie, that were detectable in both mother and neonate), and d represents the number of all species that were positive in the mother, the neonate, or both. The SIs of bifidobacteria in matched mother-infant pairs were analyzed by using analysis of variance with supplementation as the between-subject factor and time as the within-subject factor.

An α error of $<0.05\%$ was considered significant. To correct for multiple testing, the significance value for the percentage of subjects who were positive for specific bacterial species was set to 0.005 for the bifidobacteria (9 analyzed bifidobacteria species), to 0.006 for the lactobacilli (8 analyzed lactobacilli species), to 0.003 for surface markers (17 variables tested by using FACS), and to 0.005 for the cytokines (10 cytokines measured by using the Bio-Plex flow cytometric assay). Because of the exploratory nature of this study, we did not wish to exclude any important relations by using stringent correction factors for multiple analyses. However, we recognize the potential for type 1 error, and data have been interpreted conservatively in this respect. Accordingly, our discussion has focused on differences that are most likely to be of biological significance (namely, differences of $P < 0.001$).

RESULTS

Study population

Two hundred pregnant women were assessed for eligibility (**Figure 1**). Of this group, 75 women were not interested in participating in the study. Seventy-seven women did not fulfill the inclusion criteria: 37 planned to deliver at other hospitals, 22 wanted to donate cord blood stem cells and hence could not provide cord blood, and 18 required medication. Thus, 48 women were randomly assigned to 1 of the 2 supplementation groups. Seven women dropped out for personal or medical reasons: delivery in another hospital ($n = 2$), moving to another city ($n = 1$), complications during pregnancy ($n = 1$), gestational diabetes ($n = 2$), or antiinflammatory medication ($n = 1$). Five women were excluded because of noncompliance with the study procedures, which was unrelated to the supplement assignment. Three other women reported nontolerance of the supplementation: one woman from the GOS/lcFOS group had constipation and bloating, and 2 women from the placebo group had diarrhea or reflux. Thus, 33 women completed the study (n = 17 and 16 in the GOS/lcFOS and placebo groups, respectively; dropout rate of 31%).

Study group characteristics did not differ significantly between the 2 groups at study entry or at delivery (**Table 1**). Three neonates from the GOS/lcFOS group and 2 from the placebo group were treated with antibiotics for 4–7 d during the first 3 wk of life because of suspected amnion infection syndrome.

Overall tolerance of the GOS/lcFOS and placebo (maltodextrin) supplements was good. Complaints about regurgitation, bloating, and abdominal pain were reported in the 2 groups with similar frequency. No major side effects were observed. All neonates were breastfed, but 3 neonates received infant formula in addition to breast milk during the first 3 d of life. During the first 6 mo of life, most infants from the GOS/lcFOS and placebo groups predominantly received breast milk. At the age of 6 mo, 2 infants from each group still were being exclusively fed breast milk. Two infants from the GOS/lcFOS group had atopic eczema (physician's diagnosis) at the age of 3 and 5 mo, respectively.

Effect of GOS/lcFOS on maternal gut microbiota

At study entry, the total number of bacteria in maternal samples was significantly ($P = 0.017$) lower in the GOS/FOS group than in the placebo group, whereas the baseline percentages of bifidobacteria and lactobacilli (assessed by qPCR and FISH) did

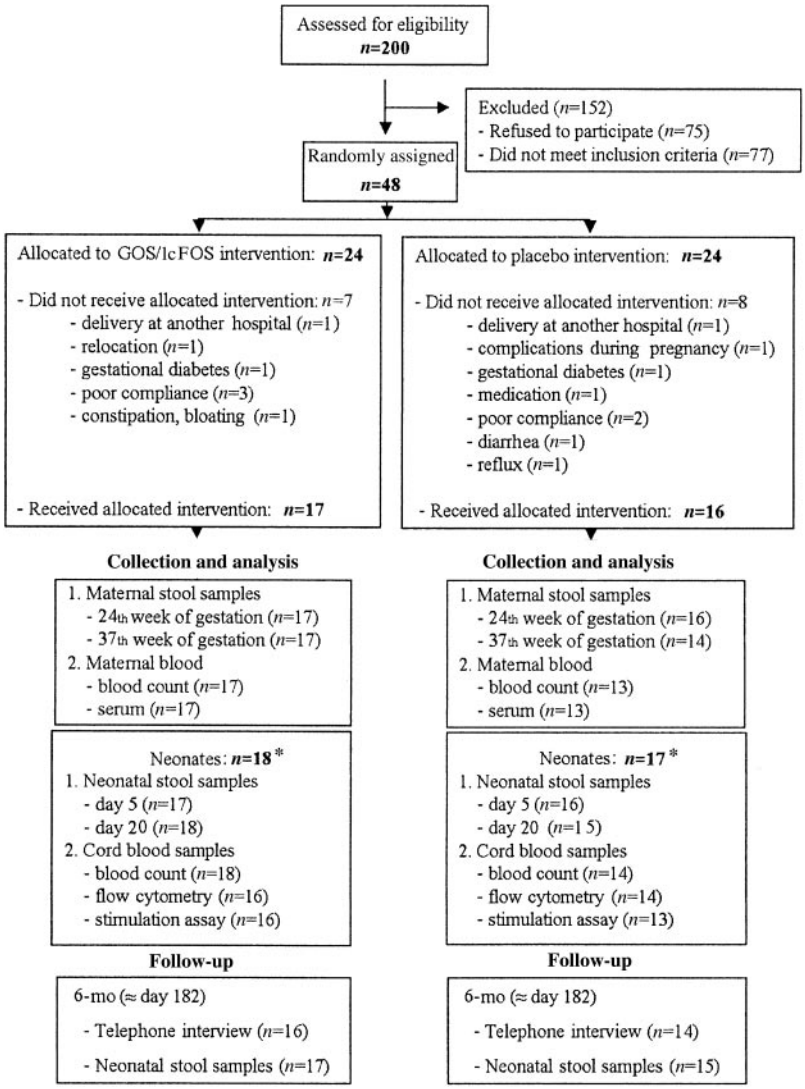

FIGURE 1. The flow chart of participant recruitment, sample collection, and follow-up. GOS, galactooligosaccharides; lcFOS, long-chain fructooligosaccharides. Stool samples were missing for various reasons. Maternal stool samples at week 37 of gestation: 2 mothers from the placebo group did not immediately freeze the stool samples after collection. Neonatal stool samples from day 5: 1 mother from the GOS/lcFOS group and one from the placebo group were unable to collect a sample from the diapers of their infants. Neonatal stool samples from day 20: 1 mother from the placebo group did not immediately freeze the neonatal stool sample after collection, and 1 sample from the placebo group did not contain enough material for analysis. Neonatal stool samples from day 182: 1 participant from the GOS/lcFOS group and 2 participants from the placebo group were lost to follow-up. Blood samples were also missing for various reasons. Maternal blood samples at delivery: 3 mothers from the placebo group delivered at other hospitals. Cord blood samples: 3 mothers from the placebo group delivered at other hospitals. Two cord blood tubes from the GOS/lcFOS group and one tube from the placebo group did not contain enough material for all analyses. Three follow-up interviews were missing because 1 woman from the GOS/lcFOS group and 2 women from the placebo group were lost to follow-up. *Each group included one twin pregnancy.

not differ significantly between the 2 groups. The percentage of bifidobacteria (determined by qPCR and FISH) was significantly higher in the GOS/lcFOS group (21.0%) than at study entry (9.2%; $P = 0.001$) and significantly higher than that in the placebo group (12.4%, $P = 0.026$) (**Figure 2**). Total bacterial counts, percentages of lactobacilli, stool consistency, stool frequency, stool pH, regurgitation, and vaginal pH did not differ between the 2 groups after supplementation (data not shown). At the single species level, considerable interindividual and intraindividual differences in bifidobacteria and lactobacilli composition were observed between the 2 groups (assessed by qPCR; data not shown). Similarly, the percentages of mothers who were positive for a certain species varied greatly (**Table 2**); *B. catenulatum*, *B. infantis*, and *L. acidophilus* were the most frequent species. After GOS/lcFOS supplementation, the percentages of GOS/lcFOS group mothers who were positive for certain bifidobacteria and lactobacilli species did not differ significantly from baseline or from the placebo group.

Effect of GOS/lcFOS on the development of the neonatal gut microbiota

The mode of delivery influences the composition of the neonatal microbiota. Therefore, neonates born by cesarean delivery were excluded from the analyses ($n = 5$ and 1 for GOS/lcFOS and placebo groups, respectively). Neonatal stool pH, the total numbers of bacteria (assessed by using 4'-6-diamidino-2-phenyl indole staining), and the percentages of bifidobacteria and lactobacilli (assessed by qPCR and FISH) on days 5, 20, and 182 did

TABLE 1
Baseline characteristics of the study group at study entry and at delivery[1]

	GOS/lcFOS group	Placebo group	P^2
Mother[3]			
Age at study entry (y)	33 (23–48)[4]	35 (22–43)	0.096
Pregnancy [*n* (%)]			0.965
Singleton	16 (94.1)	15 (93.8)	
Gemini	1 (5.9)	1 (6.3)	
Gestational age at delivery (wk)	39.57 (36.7–41.2)	40.34 (36.4–41.5)	0.083
Delivery mode [*n* (%)]			0.292
Spontaneous	11 (64.7)	14 (56.0)	
Caesarean section	4 (23.5)	1 (6.3)	
Vacuum extraction	2 (11.8)	1 (6.3)	
Allergies [*n* (%)]			
Allergic asthma	4 (23.5)	3 (18.7)	0.737
Hay fever	7 (41.2)	9 (56.3)	0.387
Seasonal allergies	6 (35.3)	8 (50.0)	0.393
Atopic eczema	6 (35.3)	5 (31.3)	0.805
Urticaria	4 (23.5)	2 (12.5)	0.412
Food allergies	6 (35.3)	4 (25.0)	0.520
Smoking [*n* (%)]			0.965
Yes	1 (5.9)	1 (6.3)	
No	16 (94.1)	15 (93.7)	
Neonate[5]			
Sex [*n* (%)]			0.61
F	8 (44.4)	8 (47.1)	
M	10 (55.6)	9 (52.9)	
APGAR score			
(5-min)	10 (10–10)	10 (10–10)	1.00
(10-min)	10 (10–10)	10 (10–10)	1.00
Umbilical pH[6]	7.34 (7.1–7.4)	7.34 (7.2–7.4)	0.511
Birth weight (g)	3368 (2345–4080)	3370 (2590–3855)	0.974
Birth length (cm)	51.0 (47.0–54.0)	52.0 (49.0–64.0)	0.13

[1] GOS, galactooligosaccharides; lcFOS, long-chain fructooligosaccharides.
[2] Differences between groups were calculated by using the Mann-Whitney *U* test for continuous data and the chi-square test for dichotomous data.
[3] $n = 17$ and 16 for GOS/lcFOS and placebo groups, respectively.
[4] Median; range in parentheses (all such values).
[5] $n = 18$ and 17 for GOS/lcFOS and placebo groups, respectively.
[6] $n = 18$ and 16 for GOS/lcFOS and placebo groups, respectively.

not differ significantly between the 2 supplementation groups (**Figure 3**). Time significantly affected the percentages of bifidobacteria ($P = 0.005$) and lactobacilli ($P = 0.012$, analysis of covariance) in neonatal stool samples. Intragroup comparisons showed a significant increase in the numbers of lactobacilli in the placebo group ($P = 0.037$, assessed by PCR; $P = 0.018$, assessed by FISH; paired Wilcoxon test) from day 20 to day 182. At the single species level, large intraindividual and interindividual variations in the numbers of bifidobacteria and lactobacilli (assessed by qPCR; data not shown) and in the percentages of neonates positive for single bifidobacterial and lactobacilli species were observed (**Table 3**). However, no significant differences were observed between the 2 groups. *B. infantis*, *B. breve*, *L. acidophilus*, and *L. paracasei* were the most prevalent species in both groups at all time points.

Bacterial diversity and similarity

After supplementation, DIs of bifidobacteria and lactobacilli in maternal and neonatal stool samples did not differ significantly between the 2 groups (**Table 4**). However, the maternal bifidobacteria DIs after supplementation significantly affected the infant bifidobacteria DIs independent of time and supplementation. Furthermore, time had a significant effect on the infant lactobacilli DIs independent of supplementation. A significant interaction between time and the maternal lactobacilli DIs after supplementation was observed in the subgroup analysis. In both groups, maternal lactobacilli DIs were significantly higher than those in the neonatal samples from day 20 ($P = 0.012$ in the GOS/lcFOS group; $P = 0.014$ in the placebo group; paired Wilcoxon test), whereas bifidobacterial diversity did not differ significantly. At all analyzed time points, stool samples from neonates in both groups had significantly higher bifidobacteria than lactobacilli DIs ($P < 0.02$, paired Wilcoxon test).

A decrease in mother-neonate SIs independent of the supplementation group was observed over time for bifidobacteria (**Table 5**) but not for lactobacilli (data not shown). However, intervention with GOS/lcFOS did not influence the mother-neonate *Bifidobacterium* and *Lactobacillus* SIs (data not shown).

Effect of GOS/lcFOS on fetal lymphocyte subsets and cytokine secretion

To test whether maternal GOS/lcFOS supplementation affects fetal immunity, we performed extensive phenotyping of lymphocyte subsets. CB samples from antibiotic-treated neonates were

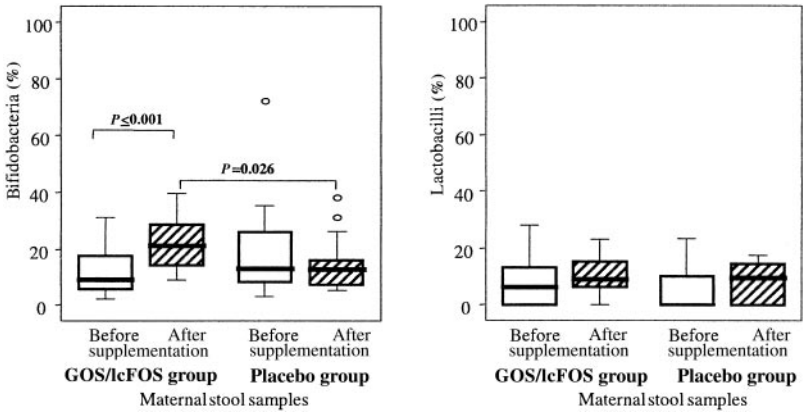

FIGURE 2. Percentages of bifidobacteria (left) and lactobacilli (right) in maternal stool samples. GOS, galactooligosaccharides; lcFOS, long-chain fructooligosaccharides. Bacteria were quantified by using quantitative polymerase chain reaction. $n = 17$ before and after supplementation, respectively, in the GOS/lcFOS group and 16 and 14 before and after supplementation, respectively, in the placebo group. Black bar, median; boxes, interquartile range; T-bars, range; ○, outliers (between 1.5 and 3 interquartile ranges from the end of a box). Differences between groups were assessed by using the Mann-Whitney *U* test; differences within one group before and after supplementation (intragroup comparisons) were assessed by using the paired Wilcoxon test. Percentages of bifidobacteria before supplementation significantly affected the percentages of bifidobacteria after supplementation ($P = 0.005$, univariate ANCOVA with supplementation as the factor and percentages of maternal bifidobacteria or lactobacilli before supplementation as the covariate).

excluded from the analyses. $CD4^+$ Th cells and $CD8^+$ T cytotoxic (Tc) cells predominantly (>84%) expressed the naïve ($CD45RA^+$) phenotype, whereas <13% expressed the memory marker $CD45RO^+$. The ratio of $CD4^+$ to $CD8^+$ ($CD4^+$:$CD8^+$) and the percentages of $CD4^+CD45RA^+$, $CD8^+CD45RA^+$, $CD4^+CD45RO^+$, and $CD4^+CD45RO^+$ did not differ significantly between the 2 groups (See Table S1 under "Supplemental data" in the current online issue at www.ajcn.org.). CKRs control lymphocyte migration into lymphatic and inflamed tissues. CXCR4 and CCR7 are found on naïve T cells. CXCR3 and CCR5 are characteristic of Th1 cells, whereas CCR4 is expressed on Th2 cells. In contrast to the high frequency of $CCR7^+$ cells (>87.3%), all other CKRs were expressed at a very low frequency on $CD4^+$ or $CD8^+$ T cells. CXCR4 was expressed at a high molecular density on both $CD4^+$ and $CD8^+$ T cells, whereas CXCR3 was expressed only on $CD8^+$ cells. Frequencies of

TABLE 2
Percentage of mothers who were positive for the specific *Bifidobacterium* and *Lactobacillus* species[1]

	GOS/lcFOS group		Placebo group	
	Before supplementation ($n = 17$)	After supplementation ($n = 17$)	Before supplementation ($n = 16$)	After supplementation ($n = 14$)
	%		%	
B. adolescentis	29	47	38	50
B. angulatum	12	18	19	27
B. animalis	12	24	0	0
B. bifidum	18	24	25	43
B. breve	35[2]	35	6	43
B. catenulatum	71	71	50	64
B. dentium	6	0	6	0
B. infantis	59	71	56	72
B. longum	24	12	6	7
L. acidophilus	59	77	31	43
L. casei	24[3]	6	0	0
L. delbrueckii	12	35	13	21
L. fermentum	35	12	19	36
L. paracasei	41[4]	41	6	14
L. plantarum	0	0	19	21
L. reuteri	12	18	0	7
L. rhamnosus	12	18	19	14

[1] GOS, galactooligosaccharides; lcFOS, long-chain fructooligosaccharides. Bacteria were quantified in the maternal stool samples taken before and after supplementation with the use of quantitative polymerase chain reaction. There were no significant differences between time points within a supplementation group based on the McNemar test. No significant main effects or interactions were observed in a general log linear model with percentages of mothers positive for a specific bacterial species, supplementation, and time included as factors.

[2–4] Significant differences between groups before supplementation (chi-square test): [2]$P = 0.04$, [3]$P = 0.038$, [4]$P = 0.019$. Differences were no longer significant after correction for multiple testing.

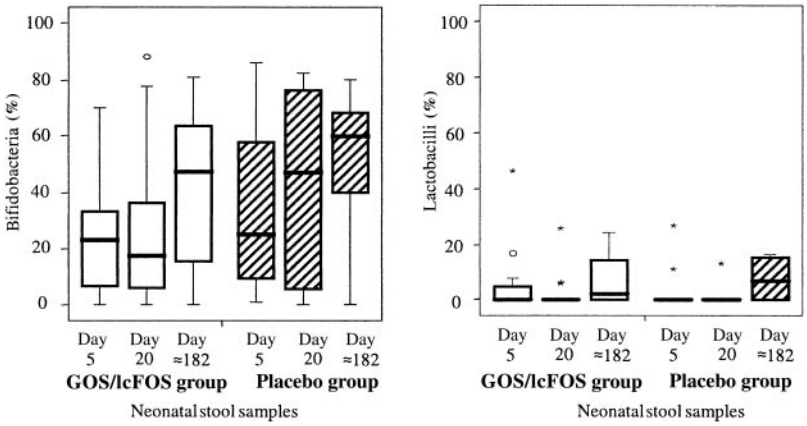

FIGURE 3. Percentages of bifidobacteria (left) and lactobacilli (right) in neonatal stool samples. GOS, galactooligosaccharides; lcFOS, long-chain fructooligosaccharides. Bacteria were quantified by using quantitative polymerase chain reaction. n = 12, 13, and 1 on days 5, 20, and ≈182, respectively, in the GOS group and 15, 14, and 14 on days 5, 20, and ≈182, respectively, in the placebo group. Black bar, median; boxes, interquartile range; T-bars, range; ○, outliers (between 1.5 and 3 interquartile ranges from the end of a box). *Extreme values, ie, >3 interquartile ranges from the end of a box. Differences between groups were assessed by using the Mann-Whitney *U* test; differences within one group before and after supplementation (intragroup comparisons) were assessed by using the paired Wilcoxon text. Time significantly affected the percentages of bifidobacteria ($P = 0.005$) and lactobacilli ($P = 0.012$) in infant stool samples. No other main effects or interactions were observed (ANCOVA with supplementation as the between-subject factor and time as the within-subject factor, and percentages of material bifidobacteria or lactobacilli after supplementation as the covariate).

CKR^+ lymphocytes within $CD4^+$, $CD8^+$, $CD4^+CD45RA^+$, and $CD8^+CD45RA^+$ CB T cell subsets did not differ significantly between the 2 groups. The early and late activation markers CD69 and CD25 were expressed at similar levels on the different $CD4^+$ subsets in both groups (data not shown). No significant difference in the frequency of $CD4^+CD25^{high}$ regulatory T cells was observed.

Cytokine and chemokine secretion patterns were determined in supernatants of whole-CB cultures stimulated with mitogen and allergens. Proinflammatory (ie, tumor necrosis factor-α, IL-1, IL-6, IL-8, monocyte chemoattractant protein-1, and macrophage-inhibiting protein-1β), Th1- and Th2-related (ie, interferon-γ, IL-2, and IL-4), and regulatory (IL-10) cytokines did not differ significantly between the 2 groups (data not shown).

TABLE 3
Percentage of neonates who were positive for specific *Bifidobacterium* and *Lactobacillus* species[1]

	GOS/lcFOS group			Placebo group		
	Day 5 ($n = 12$)	Day 20 ($n = 13$)	Day 182 ($n = 12$)	Day 5 ($n = 15$)	Day 20 ($n = 14$)	Day 182 ($n = 14$)
		%			%	
B. adolescentis	17	23	17	33	21	14
B. angulatum	8	0	0	7	7	0
B. animalis	0	0	0	0	0	0
B. bifidum	25	31	33	60	43	50
B. breve	67	69	75	80	71	86
B. catenulatum	33	39	25	40	21	7
B. dentium	0	0	0	0	0	0
B. infantis	100	77	75	100	86	93
B. longum	17	23	25	27	14	21
L. acidophilus	42	31	50	20	7[2]	64[2]
L. casei	17	8	25	7	7	21
L. delbrueckii	0	0	0	7	0	0
L. fermentum	25	15	8	13	14	21
L. paracasei	33	15	25	20	7	36
L. plantarum	17	15	8	7	0	0
L. reuteri	0	0	0	0	0	0
L. rhamnosus	0	0	0	0	0	0

[1] GOS, galactooligosaccharides; lcFOS, long-chain fructooligosaccharides. Bacteria were quantified in neonatal stool samples from day 5, day 20, and day ≈182 by using quantitation polymerase chain reaction. Differences between supplementation groups were calculated by using a chi-square test. Differences between the 3 sampling time points within one supplementation group were calculated by using McNemar's test. No significant main effects or interactions were observed (general log linear model with percentages of infants positive for a specific bacterial species, supplementation, and time as factors).

[2] $P = 0.016$, but no longer significantly different after correction for multiple testing.

TABLE 4
Bifidobacteria and lactobacilli diversity indexes (DIs) in mothers receiving galactooligosaccharides and long-chain fructooligosaccharides (GOS/lcFOS) or placebo supplementation and in their neonates[1]

	Bifidobacteria[2]			Lactobacilli[3]		
	GOS/lcFOS group	Placebo group	*P*	GOS/lcFOS group	Placebo group	*P*
Mother after supplementation						
n[4]	17	14		17	14	
DI (%)	22 (11–77)[5]	33 (11–55)	0.670	22[a] (0–44)	22[a] (0–44)	0.289
Neonate						
Day 5						
n	12	15		12	15	
DI (%)	33 (11–55)	33 (22–55)	0.188	0 (0–50)	0 (0–63)	0.527
Day 20						
n	13	14		13	14	
DI (%)	22 (0–55)	33 (0–55)	0.861	0 (0–44)[b]	0 (0–33)[b]	0.186
Approximately day 182						
n	12	14		12	14	
DI (%)	28 (0–44)	33 (11–55)	0.811	6 (0–33)	22 (0–33)	0.660

[1] Maternal samples were taken after supplementation; neonatal stool samples were taken on days 5, 20, and ≈182. Differences between supplementation groups were calculated with Mann-Whitney *U* test. Medians with different superscript letters are significantly different: $P = 0.012$ for GOS/lcFOS, $P = 0.014$ for placebo (paired Wilcoxon test).

[2] The effect of maternal bifidobacterial DIs after supplementation on infant bifidobacterial DIs was significant, $P = 0.001$. There were no other significant main effects or interactions (ANCOVA with supplementation as between-subject factor, time as within-subject factor, and maternal DI for bifidobacteria after supplementation as covariate).

[3] The effect of time on infant lactobacilli DIs ($P = 0.001$) and the time × maternal lactobacilli DI interaction ($P = 0.002$) after supplementation were significant. There were no other main effects or interactions (ANCOVA with supplementation as between-subject factor, time as the within-subject factor, and maternal DI for lactobacilli after supplementation as the covariate).

[4] Number of analyzed samples at each time point in each group.

[5] Median; range in parentheses (all such values).

DISCUSSION

To our knowledge, this trial is the first randomized clinical trial to examine the long-term effects of GOS/lcFOS supplementation during pregnancy on the maternal and neonatal gut microbiota and also to assess possible effects on the neonatal immune system. Consistent with previous findings in adults (34–37), daily supplementation with 3 doses of 3 g GOS/lcFOS in the last trimester of pregnancy was well tolerated by pregnant women in the present study, and it significantly increased the numbers of bifidobacteria but not of lactobacilli. Intake doses of FOS that had bifidogenic effects in adults ranged from 4 to 15 g/d (ie, 0.05–0.11 $g \cdot kg^{-1} \cdot d^{-1}$), and they were observed after only 2–5 wk, independent of chain length or GOS or FOS type (34–37). Whereas some studies reported effects on bowel habits (34, 36, 38, 39), we found no effects of GOS/lcFOS supplementation on bowel habits, stool frequency or consistency, or stool or vaginal pH. Differences might be attributed to variations among the studies in daily prebiotic intake, study population, duration of supplementation, and extrinsic factors such as diet, hormone status, or physical activity. In the present study, *B. catenulatum* was the most prevalent and *B. infantis* and *B. adolescentis* were the next most prevalent species in maternal stool samples, which is consistent with the findings of other studies (40–43). We observed considerable interindividual and intraindividual differences in microbial bifidobacteria and lactobacilli microbiota composition in the 2 groups. As was found in other studies (44,

TABLE 5
Similarity index of bifidobacteria in matched mother-neonate pairs[1]

	Mother after supplementation–neonate day 5			Mother after supplementation–neonate day 20			Mother after supplementation–neonate ≈day 182		
	GOS/lcFOS group	Placebo group	*P*	GOS/lcFOS group	Placebo group	*P*	GOS/lcFOS group	Placebo group	*P*
Bifidobacteria			0.154			0.439			0.178
n[2]	12	14		13	14		12	14	
Similarity index (%)	50 (0–100)[3]	60 (25–100)		33 (0–67)	50 (0–100)		33 (0–60)	45 (0–100)	

[1] GOS, galactooligosaccharides; lcFOS, long-chain fructooligosaccharides. Samples were taken from the mothers after supplementation and from the neonates on day 5, day 20, and ≈day 182. The effect of time was significant, $P = 0.004$. There were no other main effects or interactions (ANOVA with supplementation as between-subject factor and time as within-subject factor).

[2] The number of samples analyzed at each time point in each group.

[3] Median; range in parentheses (all such values).

45), the predominant bacterial community of each subject in the present study was host-specific and stable. However, depending on the subject, high fluctuations in the numbers of species and in the strain composition can occur over time (42, 46, 47). Intergroup and intragroup comparisons showed no selective stimulation of a particular bifidobacteria or lactobacilli species by GOS/lcFOS. However, the present study was probably underpowered to detect significant differences at the single species level. Nevertheless, the lack of a selective stimulation of bifidobacteria or lactobacilli indicates a stronger effect of the indigenous microbiota and shows that the microbiota in adults remains rather constant, despite changes in dietary intake. Most other trials directly supplemented term or preterm infants with prebiotics and compared their microbiota with that of infants fed standard formula (48–51). Because all neonates in the present study were breastfed, we searched for studies that included breastfed infants as a control group (19–21, 52). However, different time points for stool sampling and different quantification techniques made direct comparisons with other studies difficult. In the present study, neither the total bacterial counts nor the percentages of bifidobacteria and lactobacilli showed any significant differences between the 2 groups at any time point, but bifidobacteria and lactobacilli increased over time. The GOS/lcFOS-induced increase in maternal bifidobacterial numbers did not influence the bifidobacterial percentages of the neonates.

In agreement with previous studies (20, 21), we found that *B. infantis* belonged to the most dominant species, and that *B. breve*, *B. longum*, *L. acidophilus*, and *L. paracasei* were the next most dominant species in 5- and 20-d-old breastfed neonates. After 6 mo, the most frequent species in the present study were *B. infantis*, *B. breve*, *L. acidophilus*, and *L. paracasei*, whereas *B. angulatum*, *B. animalis*, *B. dentium*, *L. delbrueckii*, and *L. rhamnosus* were not detectable. These findings are in line with the study by Haarman and Knol (20, 21), but they contrast with the results of Ahrne et al (53), who found that *L. rhamnosus* was the most commonly isolated lactobacillus strain (21%) in 6-mo-old breastfed Swedish children. Again, methodologic differences (detection sensitivity), country-dependent differences, and genetic variations may contribute to the observed differences in neonatal microbiota.

In neonates from both groups in the present study, the DIs for bifidobacteria were always higher than those for lactobacilli. This finding is in accordance with results from studies showing that bifidobacteria dominate lactobacilli in this age group (10, 54). The complexity of bifidobacteria and lactobacilli in neonates of mothers from both groups were comparable, as shown by similar DIs and SIs and by the increases in neonatal lactobacilli and bifidobacterial complexity over time. Maternal bifidobacterial diversity significantly affected neonatal bifidobacterial diversity. Thus, whereas the amount of total maternal bifidobacteria is not relevant for the transfer to the neonate, maternal bifidobacterial diversity seems to be more important. The SIs between mothers and neonates were high in the first week of life and decreased significantly during the study period. This suggests that the maternal microbiota plays a role in the first colonization, whereas other factors (eg, the specific diet) influence the neonatal microbiota later on. Moreover, 50–70% of the bifidobacteria are apparently not detected by the set of primers and probes used in the present study (16, 20, 21). Satokari et al (40) showed that several of the novel bifidobacteria should be characterized.

For a secondary endpoint, we tested the effect of maternal GOS/FOS intervention on the fetal immune system. Only a few human studies have investigated the effects of prebiotics alone or in combination with other dietary supplements on immune competence. Prebiotic supplementation resulted in minor changes in systemic immune functions, such as decreases in phagocytic activity (55). Because in vitro manipulation affects T cell function, cytokine production, and surface molecule expression (56), we undertook a comprehensive exploratory examination of whole-CB immune parameters to obtain a more accurate picture of the in vivo situation (57). Previous studies showed that increasing antigenic exposure from the maternal environment via transplacental passage (58–60) leads to activation and differentiation of Th1 or Th2 cells (61). However, the analyses of a broad spectrum of Th1/Tc1- and Th2/Tc2-related CKRs on $CD4^+$ and $CD8^+$ CB T cells and of cytokine secretion patterns found no significant differences between the 2 groups.

Similarly, cytokine responses in CB samples showed no significant differences between the 2 groups. Thus, an effect of maternal GOS/lcFOS supplementation with subsequent bifidoenriched maternal gut microbiota on fetal immune parameters is unlikely. Children born by cesarean delivery have a greater risk of developing allergies later in life. Whether the immune systems of these children may benefit indirectly from a maternal bifidobacteria-enriched flora remains to be determined. However, immune parameters for children born by cesarean delivery in the present study did not appear to differ significantly from those of the other children. Because of sample volume limitations, other immune parameters—eg, phagocytic and natural killer cell activity—were not assessed in the present study.

In conclusion, this study shows that long-term dietary intervention with GOS/lcFOS (ratio 9:1) at the given dosage (3 times/d at 3 g) was well tolerated by pregnant women, and it significantly increased the proportions of bifidobacteria in the maternal gut. Our results further suggest that the maternal microbiota plays a role in the initial colonization of the infant gut during the first days of life, as assessed by a high SI (60%) between mother and child. However, we did not observe a direct effect on the bacterial transfer between mother and child as a result of the GOS/lcFOS supplementation, and this lack of direct effect is most likely due to a masked effect of the human oligosaccharides found in the breast milk. For ethical reasons, it is not possible to set up a study population selecting a priori for mothers who intend not to breastfeed. Furthermore, the rate of breastfeeding is very high in Germany (62). Maternal supplementation seems to be safe for the neonate, because fetal immune parameters were not affected. It remains to be determined whether and to what extent formula-fed neonates or neonates born by cesarean delivery could benefit from a bifidobacteria-enriched maternal microbiota.

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The authors' responsibilities were as follows—RS, DR-D, and SK-E: data and sample collections; RS: analysis of fetal immune parameters; MH and JK: maternal and neonatal microbiota analyses; WT: statistical expertise; RS and SK-E: responsibility for the integrity and accuracy of the database and the

statistical analysis; SK-E and BK: the concept and design of the study; RS and SK-E: interpretation of the data, together with MH, JK, WT, CB, DJS, and BK; RS: the manuscript draft; and all authors: critical review of the manuscript. BVK and SK-E share senior authorship of the manuscript. None of the authors had a personal or financial conflict of interest.

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