ASN EST. 1928

Randomized controlled trial on the impact of early-life intervention with bifidobacteria on the healthy infant fecal microbiota and metabolome

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ABSTRACT

Background: Early-life colonization of the intestinal tract is a dynamic process influenced by numerous factors. The impact of probiotic-supplemented infant formula on the composition and function of the infant gut microbiota is not well defined.

Objective: We sought to determine the effects of a bifidobacteria-containing formula on the healthy human intestinal microbiome during the first year of life.

Design: A double-blind, randomized, placebo-controlled study of newborn infants assigned to a standard whey-based formula containing a total of 10^7 colony-forming units (CFU)/g of *Bifidobacterium bifidum*, *Bifidobacterium breve*, *Bifidobacterium longum*, *B. longum* subspecies *infantis* (intervention), or to a control formula without bifidobacteria (placebo). Breastfed controls were included. Diversity and composition of fecal microbiota were determined by 16S ribosomal RNA gene amplicon sequencing, and metabolite profiles were analyzed by ultrahigh-performance liquid chromatography–mass spectrometry over a period of 2 y.

Results: Infants (n = 106) were randomly assigned to either the interventional (n = 48) or placebo (n = 49) group; 9 infants were exclusively breastfed throughout the entire intervention period of 12 mo. Infants exposed to bifidobacteria-supplemented formula showed decreased occurrence of *Bacteroides* and *Blautia* spp. associated with changes in lipids and unknown metabolites at month 1. Microbiota and metabolite profiles of intervention and placebo groups converged during the study period, and long-term colonization (24 mo) of the supplemented *Bifidobacterium* strains was not detected. Significant differences in microbiota and metabolites were detected between infants fed breast milk and those fed formula ($P < 0.005$) and between infants birthed vaginally and those birthed by cesarean delivery ($P < 0.005$). No significant differences were observed between infant feeding groups regarding growth, antibiotic uptake, or other health variables ($P > 0.05$).

Conclusion: The supplementation of bifidobacteria to infant diet can modulate the occurrence of specific bacteria and metabolites during early life with no detectable long-term effects. This trial was registered at germanctr.de as DRKS00003660. *Am J Clin Nutr* 2017;106: 1274–86.

Keywords: infant gut microbiota, probiotics, bifidobacteria, breastfeeding, 16S rRNA gene, metabolomics

INTRODUCTION

Colonization of the infant gastrointestinal tract is a dynamic process influenced by dietary and medical factors (1, 2). The effects of initial feeding regimens have been investigated in detail, clearly discriminating microbial succession in the gut of breastfed and formula-fed infants (3, 4). Although commercially available formulas are supplemented with bacteria considered as probiotics, little is known about their ability to modulate the infant gut microbial composition and function (5, 6). The vast majority of published studies have focused on the use of probiotics in disease-related contexts, including necrotizing enterocolitis in preterm infants, gastrointestinal and respiratory infections, and allergic reactions (7–10). Their use in healthy cohorts is common, but available data are limited in terms of repeated measurements over time and duration of probiotic administration (11–13). In addition to the compositional analysis

Töpfer GmbH (Dietmannsried, Germany) Töpfer GmbH, a company that manufactures and sells baby products, provided financial support and provided the infant formula to the academic institution in a blinded manner. Cell Biotech Co. Ltd. provided purified *Bifidobacterium* strains.

Supplemental Material, Supplemental Figures 1–5, and Supplemental Tables 1–3 are available from the "Online Supporting Material" link in the online posting of the article and from the same link in the online table of contents at http://ajcn.nutrition.org.

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Abbreviations used: B, exclusively breastfed; BF, breast- and formula-fed; BF+, breast- and interventional formula-fed; BF−, breast- and placebo formula-fed; F, exclusively formula-fed; F+, interventional formula; F−, placebo formula; HMO, human milk oligosaccharide; LNFP, lacto-N-fucopentaose; MS, mass spectrometry; OPLS-DA, orthogonal partial least squares discriminant analysis; OTU, operational taxonomic unit; Q-ToF, quadrupole–time of flight; rRNA, ribosomal RNA; SCFA, short-chain fatty acid; UHPLC, ultra HPLC.

Received March 27, 2017. Accepted for publication August 8, 2017.

First published online September 6, 2017; doi: https://doi.org/10.3945/ajcn.117.157529.

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of the infant microbiota, metabolic profiling contributes to the functional understanding of the intestinal ecosystem, but data from healthy infant cohorts with probiotic intervention are absent.

In this study, we analyzed the impact of infant formula supplemented with 4 *Bifidobacterium* strains on structural [16S ribosomal RNA (rRNA) gene amplicon analysis] and functional [metabolomics via ultra HPLC–mass spectrometry (UHPLC-MS)] changes in the healthy infant gut from birth through the first year of life, also including follow-up data at 2 y of age for assessment of long-lasting effects of intervention.

METHODS

Participants

Healthy term infants born by vaginal or cesarean delivery and breastfed, formula-fed, or mixed-fed (breast and formula in parallel) were enrolled in the study. Exclusion criteria were preterm births (gestational age <36 wk), high-risk pregnancies, maternal chronic illnesses (e.g., type 1 or 2 diabetes, inflammatory bowel disease), maternal mental and psychosomatic diseases, maternal BMI (in kg/m^2) <18.5 and >30, and antibiotic therapy during the final 2 mo of pregnancy. Both parents were asked to consent to participation. The targeted minimum number of infants was 80.

Study design

The study was double-blind, randomized, and placebo controlled. Block randomization with a block size of 8 was used. Participants and study staff were blinded from the beginning of the study until analyses were completed. From birth to 12 mo of age, participants were regularly provided with a control, whey-based formula (provided by Töpfer GmbH) or an interventional formula (control formula plus a total concentration of 10^7 CFU/g with equal amounts of *Bifidobacterium bifidum* BF3, *Bifidobacterium breve* BR3, *Bifidobacterium longum* subspecies *infantis* BT1, and *B. longum* BG7) by a staff member. Preparation of the formula was done at home by the parents according to the provider's instructions: Tap water was boiled and cooled to 50°C. The required amount of powdered formula (dependent on infant age) was added to the water, and the formula was mixed by shaking before being administered to the infant. When prepared according to the instructions, both of the formulas contained (per 100 mL) 281 kJ, 67 kcal, 1.7 g protein, 7.4 g carbohydrates, and 3.5 g fat, and vitamins, minerals, choline, inositol, trace elements, and dietary fiber. Total viable cell numbers in the supplemented formula were monitored by the provider via cultivation on *Bifidobacterium*-selective agar and counts were confirmed to be 10^7 CFU/g throughout the study period. On the parents' own initiative, infants were either exclusively breastfed or exclusively fed one of the study formulas as per randomization (nonlactating mothers). In the case of mixed feeding, the amount of formula given to the infant and the duration of breastfeeding were determined by the parents themselves and recorded on monthly questionnaires. The primary outcome measure was fecal microbiota dynamics during the first year of bifidobacteria supplementation, and the secondary outcome measure was fecal metabolite profiling. The trial was registered at the German Clinical Trials Register under number DRKS00003660 and the protocol was approved by the ethics committee of the medical faculty of the Technical University of Munich (approval number 5324/12). All clinical aspects of the study were supervised by a physician.

Fecal sample collection

Infant fecal samples were collected monthly throughout the first year of age (12 mo postnatally). One additional sample was collected at 2 y of age with parental consent. Samples were taken directly from diapers into sterile plastic collection tubes by the parents themselves, preferably in the morning. Tubes were then placed in sealed plastic bags alongside an oxygen-absorbing sachet to generate anaerobic conditions. Samples were stored at 4°C and transported to the laboratory within 1 d. Samples (∼100-mg aliquots) were stored at −80°C until processing. In addition, mothers were asked to provide a breast milk sample 1 mo after delivery on a voluntary basis. Samples were obtained through a milk pump and kept at 4°C for a maximum duration of 4 h until final storage as 1-mL aliquots at −80°C. Infant milk consumption, weight gain and growth, occurrence of illnesses, medication administration, and feeding characteristics were documented by the parents with questionnaires.

High-throughput sequencing of 16S rRNA gene amplicons

Metagenomic DNA was extracted from fecal aliquots thawed on ice and resuspended in 600 μL DNA stabilization buffer (Stratec Biomedical) and 400 μL phenol/chloroform/isoamyl alcohol (25:24:1, by vol; Sigma-Aldrich). Cells were mechanically lysed (3 × 6.5 m/s for 40 s) with 500 mg 0.1-mm glass beads (Roth) through the use of a bead-beater (MP Biomedicals) fitted with a cooling adapter. After heat treatment (95°C, 8 min) and centrifugation (16,000 × *g*; 5 min; 4°C), 150 μL supernatant was incubated with 15 μL ribonuclease (0.1 μg/mL; Amresco) at 37°C and centrifuged (550 × *g*; 30 min). DNA was purified with the NucleoSpin gDNA Clean-up Kit (Macherey-Nagel), following the manufacturer's instructions. Concentrations and purity were determined with the NanoDrop system (Thermo Fisher Scientific). If not processed immediately, samples were stored at −20°C. Preparation of amplicon libraries (V3–V4 region) and sequencing was performed as described in detail previously (14). After purification with the AMPure XP system (Beckmann), sequencing was carried out in paired-end mode (PE275) with pooled samples containing 25% (vol:vol) PhiX standard library in a MiSeq system (Illumina Inc.) prepared according to the manufacturer's instructions.

16S rRNA amplicon data processing and statistics

Data were analyzed as described previously (14). Raw reads were processed with the in-house–developed pipeline Integrated Microbial Next Generation Sequencing (15) based on the UPARSE approach (16). In brief, sequences were demultiplexed, trimmed to the first base with a quality score <3, and then paired. Sequences with <380 and >420 nucleotides and paired reads with an expected error >3 were excluded from the analysis. Remaining reads were trimmed by 10 nucleotides on each end to prevent analysis of the regions with distorted base composition observed at the start of sequences. The presence of

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chimeras was tested with UCHIME (17). Operational taxonomic units (OTUs) were clustered at 97% sequence similarity, and only those with a relative abundance >0.5% in ≥1 sample were kept. Taxonomies were assigned at 80% confidence level with the RDP classifier (18). Further analysis was performed in the R programming environment with the use of Rhea (19). All details of the analysis and the scripts are available online (https://lagkouvardos.github.io/Rhea/). OTU tables were normalized to account for differences in sequence depth via simple division to their sample size and then multiplication by the size of the smaller sample. OTU tables of all study groups are provided (**Supplemental Material**). β-Diversity was computed based on generalized UniFrac distances (20). α-Diversity was assessed on the basis of species richness and Shannon effective diversity (21) as explained in detail in Rhea (see GitHub link). *P* values were corrected for multiple comparisons according to the Benjamini-Hochberg method. Only taxa with a prevalence of ≥30% (proportion of samples positive for the given taxa) in one given group were considered for statistical testing. Raw sequence data are available at the European Nucleotide Archive under study accession number ERP023432.

Detection of the supplemented *Bifidobacterium* strains

Genomes of the 4 strains were provided by Cell Biotech Ltd. Unique genes in the bifidobacteria strains were identified by comparing the annotated genome with other available genomes in the Joint Genome Institute database (with the use of the Phylogenetic Profiler for Single Genes tool in the Integrated Microbial Genomes database) (22). From this analysis, specific genes, as outlined in **Supplemental Table 1**, were selected as strain-specific target genes, and putative primer pairs were designed with Primer 3 software (23). Candidate primers were evaluated for hairpin and dimer formation with Netprimer (Premier Biosoft International). The selected primers were validated in silico by performing a search with the Basic Local Alignment Search Tool against the National Center for Biotechnical Information database. Primers were also evaluated with polymerase chain reaction by testing all primer pairs against the target probiotic strains, the type strain of each species, and all other strains available at the German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH). Polymerase chain reaction was run in a thermocycler (Biometra): 95°C, 1 min; 30 cycles of 15 s at 95°C, 15 s at 60°C, and 10 s at 72°C; and 72°C for 10 min. Each reaction was performed in 20-μL batches with 10 μL ready-to-use MyTaqTM Mix (Bioline), 0.4 μL forward primer (Sigma Aldrich), 0.4 μL each of the forward and reverse primer (20μM; Sigma Aldrich), and 1 μL genomic DNA (10 ng) filled to ≤20 μL with sterile water. In addition to the target probiotic strains, primer specificity was tested as follows: *B. bifidum* BF3-specific primer against *B. bifidum* DSM20082, *B. bifidum* DSM 20215, and *B. bifidum* DSM20239; *B. breve* BR3-specific primer against *B. breve* DSM20091; *B. longum* subspecies *infantis* BT1-specific primer against *B. longum* subspecies *infantis* DSM20090; and *B. longum* subspecies *infantis* DSM20218 and *B. longum* BG7-specific primer against *B. longum* DSM 20097. Detection limits were determined by adding a defined number of each target bacterium separately to fecal samples negative for the particular strain. Additionally, DNA from pure milk powder (1 g) was extracted according to the previously mentioned procedure to confirm presence in the formula provided. Furthermore, formula was plated on Bifidobacterium Selective Medium agar to validate the presence of viable cells in the milk provided. A total of 20 colonies/strain was sampled and strain-specific analysis was performed according to the aforementioned procedure.

Metabolite analysis

Fecal aliquots were centrifuged for 60 min (12,000 × *g*; 4°C) and 50-mg pellets were homogenized with 1 mL ice cold methanol [CHROMASOLV, for HPLC, ≥99.9%; Sigma-Aldrich; –4°C to –10°C) and 500 mg of ceramic beads through the use of a TissueLyser II (Qiagen; 10,000 × *g*; 5 min; 4°C). The supernatant was used for measurement on an ACQUITY UPLC system (Waters GmbH) coupled to a Bruker Daltonics maXis quadrupole–time of flight (Q-ToF) mass spectrometer. Measurements were conducted on a W. R. Grace & Co. VisionHT C18 HL 1.5 μm (150 × 2.0 mm) in randomized duplicates within 10 batches in positive electrospray ionization mode. A gradient separation was applied with the following liquid chromatography parameters: flow rate: 0.4 mL/min; and column temperature: 40°C. The total run time was 15.5 min/sample. Solvent A consisted of 5% acetonitrile plus 0.1% formic acid, and solvent B contained 100% acetonitrile plus 0.1% formic acid. 5 μL of each sample was injected in partial loop. Starting conditions of the gradient separation were 99.5% A and 0.5% B. After 1.12 min, B was increased to 99.5% within 5.3 min. This composition was retained for 3.6 min, followed by a rapid decrease to 0.5% B in 0.5 min. This composition was retained for an additional 5 min. To ensure the quality of the analyses, a quality control mixture of all samples was injected after every 10 samples. The quality control analyses were considered for the batch normalization of the data. The MS parameters were as follows: mass range: *m/z* 50–1000; dry gas: 8 L/min; dry temperature: 200°C; nebulizer gas: 2 bar. For calibration of the MS data, a 1:4 diluted Low Concentration Tune Mix (G1969-85000; Agilent) was injected between 0.1 and 0.3 min of each analysis.

Metabolite data processing and statistics

Data obtained from the positive ionization mode was processed with Genedata Expressionist Refiner MS including filtering, calibration, alignment, and peak clustering steps. Batch normalization was performed with Genedata Analyst. For further analysis and metabolite and OTU correlation, the overall and monthly data matrices were filtered by mass defect >0.9. Additionally, a 10% cutoff for zero-presence values was applied. Masses were searched against the Kyoto Encyclopedia of Genes and Genomes (24), Human Metabolome Database (25, 26), and LIPID MAPS Structure Database (www.lipidmaps.org) by the MassTRIX web server (27, 28) with a maximum error of 0.005 Dalton. *Homo sapiens* was used as a reference organism. The assignment of the mass signals with the MassTRIX web server revealed 33% of the total number of metabolites listed in databases, but the remaining 67% is unknown. In the databases, known metabolites were classified in compound classes with the use of compound IDs from the Human Metabolome Database

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and LIPID MAPS database assigned by the MassTRIX webserver. Unsupervised multivariate data analysis (principal component analysis) was conducted with SIMCA P-9.0 (Umetrics) to determine the influence of feeding and age on metabolite profiles. For metabolite/OTU correlations, both datasets were merged and evaluated through an orthogonal partial least squares discriminant analysis (OPLS-DA) with SIMCA-P 13.0.1 (Umetrics). To extrapolate the mass signals and correlated OTUs, the loadings of each time point of the OPLS-DA analysis were extracted. The most important mass signals and OTUs for each time point and group were then ranked by importance (high to low). Cross-validation ANOVA was applied to verify the robustness of each model. Indicators such as P value (to prove the significance of the different models), the goodness-of-fit R^2Y(cum), and the goodness-of prediction Q^2(cum) were reported and read as follows: month 1: $R^2(Y) = 0.94$, $Q^2 = 0.48$, $P = 6.72 \times 10^{-7}$; month 7: $R^2(Y) = 0.53$, $Q^2 = 0.38$; $P = 1.84 \times 10^{-8}$; month 12: $R^2(Y) = 0.4$, $Q^2 = 0.18$; $P = 0.0194$. To determine separation of the formula groups at month 12, an additional orthogonal component was added to the model to confirm that no further separation along the y-axis could be obtained.

Short-chain fatty acid analysis

Standards as pyruvic acid, lactic acid, propionic acid, butyric acid, valeric acid, and isovaleric acid were prepared as derivatized 1 part per million standard solutions as follows: Solutions of each standard and methanol fecal extracts were derivatized according to the AMP+ Mass Spectrometry Kit instructions (Caymen Chemicals). A total of 88 μL derivatized solution/sample and standard was diluted with 352 μL of a mixture of solvent A:B (99:1, vol:vol). The following analysis of the short-chain fatty acid (SCFA) derivatives was performed with a UHPLC/Q-ToF-MS in positive electrospray ionization mode. The SCFAs were analyzed by gradient separation. The total runtime of each analysis was 22 min plus a 2 min prerun. 1 μL of each solution was injected in partial loop. The gradient separation took place on a Waters BEH C8 column (1.7 μm, 2.1 $\times$ 150 mm). Solvent A consisted of 5 mmol/L ammonium acetate plus 0.1% acetic acid, and solvent B contained 100% acetonitrile. Starting conditions of the gradient separation were 99% A. This was retained for 1 min. Within 16 min, A was decreased from 99% A to 1% A and retained for 2 min. Within 0.2 min %A was increased again to 99% A and on hold for 2.8 min. The flow rate was 0.3 mL/min and column temperature was 40°C. For calibration purposes, a 1:4 diluted ESI-L Low Concentration Tuning Mix (Agilent) was injected in the first 0.1 min of the analysis. MS parameters were as follows: Mass range: m/z 50–1200; capillary: 4500 V; endplate offset: −500 V; nebulizer gas: 2.0 bar; dry gas: 8 L/min; dry heater: 200°C.

SCFAs were quantified through external calibration based on the extracted peak areas of each standard concentration via the calculated calibration function. Retention time was extracted with DataAnalysis Version 4.1 (Bruker Daltonics GmbH). SCFA were evaluated and quantified with QuantAnalysis Version 2.1 (Bruker Daltonics GmbH). Kruskal-Nemenyi significance test for the multiple comparisons of mean rank sums (PMCMR package, version 4.1) (29) of each SCFA in the different groups was performed with R Studio version 0.99.489.

HPLC-fluorescence analysis of human milk oligosaccharides

Human milk oligosaccharide (HMO) analysis was performed with HPLC after fluorescent derivatization at the University of California, San Diego, as previously described (30, 31). Mothers used a milk pump with a sterile, single-use container and collected 50 mL of breast milk in the morning just before nursing. The milk was immediately stored in the laboratory at 4°C and divided into aliquots within 24 h. Samples were stored at −80°C until further analysis. For HMO analysis, raffinose was added to each milk sample as an internal standard for absolute quantification. The total concentration of HMOs was calculated as the sum of the specific oligosaccharides detected. The following 16 HMOs were detected based on retention time comparison with commercial standard oligosaccharides and MS analysis: 2′-fucosyllactose, 3-fucosyllactose, 3′-sialyllactose, lacto-N-tetraose, lacto-N-neotetraose, lacto-N-fucopentaose (LNFP) I, LNFPII, LNFPIII, sialyl-LNT b and c, difucosyl-LNT, disialyl-LNT, fucosyl-lacto-N-hexaose, difucosyl-lacto-N-hexaose, fucosyl-disialyl-lacto-N-hexaose, and disialyl-lacto-N-hexaose. Secretor status was defined by the presence of 2′-fucosyllactose or LNFP1.

RESULTS

We assessed 117 healthy infants for eligibility (**Figure 1**). Of those eligible, 106 infants completed the study after enrollment, and 70 of those participants attended a 2-y follow-up. Breast milk samples for HMO analysis were donated by 27 mothers 1 mo after giving birth. Of the 106 infants who completed the study, 48 were randomly assigned to the group fed interventional formula (F+) and 49 to the group fed placebo formula (F−). Nine infants were exclusively breastfed (B) for the whole study period. Of the randomly assigned infants, 22 were exclusively formula-fed (F; F+: $n = 11$; F−: $n = 11$) and 75 were breast- and formula-fed (BF) [either breast- and interventional formula-fed (BF+) or breast- and placebo formula-fed (BF−)] throughout the first year of life. Cohort characteristics were comparable between feeding groups (**Table 1**). In particular, antibiotic treatment, age at weaning, and start of solid food intake were similar. Mode of delivery affected microbiota but not metabolite composition at months 1 and 3. No differences were measured at later ages (**Supplemental Figure 1**). Infants exposed to antibiotics (**Supplemental Figure 2**, marked in red) showed no aberrant microbiota profiles. Nonetheless, the low number of cases does not allow us to draw conclusions on the impact of antibiotics in general. It is also important to mention that, in most cases, the time interval between antibiotic treatment and fecal sampling was several weeks, most likely reverting to the normal microbiota. No adverse effects were reported for F+, and size and weight were similar to that of F− infants (**Supplemental Figure 3**).

Bifidobacteria supplementation did not compensate for differences in gut microbial profiles between F and B infants

We first assessed changes in fecal bacterial community structure in response to bifidobacteria-supplemented and placebo formula at months 1, 12, and 24, demonstrating that β-diversity

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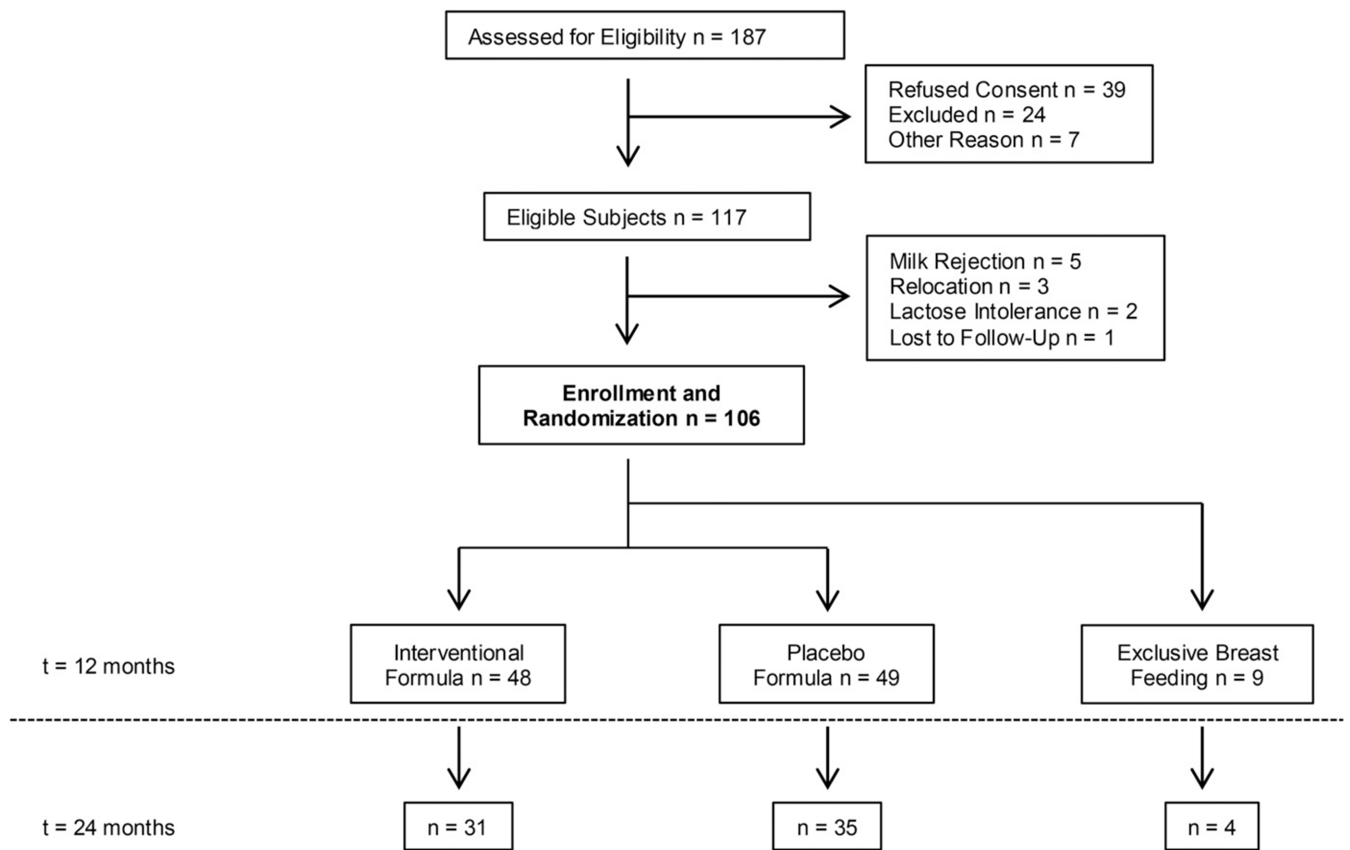

FIGURE 1 Scheme of participant enrollment and study progress. Participants were randomly administered formula with (*n* = 48) or without (*n* = 49) bifidobacteria, which was provided to the infants either exclusively or in parallel to breast milk; 9 infants were breastfed for the entire study period. Of the 106 infants who completed the study, 70 were observed at the age of 2 y.

differed between B and F infants at months 1 and 12 despite marked interindividual differences (**Figure 2**A). Analysis of infants at 2 y of age showed no effect of initial feeding on the overall microbiota phylogenetic makeup. Importantly, no major differences were observed between F+ and F− infants at any measured time point. Richness and Shannon effective counts (Figure 2B, C) were significantly lower in B infants throughout the intervention. Consistent with the dynamics in β-diversity, differences in α-diversity between the groups disappeared at 2 y of age. Relative abundance of bifidobacteria was highest in B infants, with significant effects of BF+ at months 3 and 7 (Figure 2D). However, this difference might be confounded by a higher consumption of breast milk (formula uptake per day at month 3: BF+: 115 ± 97 mL, BF−: 215 ± 124 mL, $P = 0.03$; at month 7: BF+: 78 ± 41 mL, BF−: 96 ± 62 mL, $P = 0.3$). Hence, we cannot exclude the role of HMOs in the selection of bifidobacteria under these mixed feeding conditions. The impact of breast milk on community composition continuously declined with advanced age, reaching a relative abundance of bifidobacteria similar to that found in F infants at month 24 (ranging around 10% median relative abundance).

Decreased detection of *Bacteroides fragilis* and *Blautia* species was associated with bifidobacteria supplementation

To further specify the effect of bifidobacteria supplementation, we analyzed fecal samples at months 1, 3, 5, 7, 9, and 12 in F+ (*n* = 11) compared with F− (*n* = 11). We used B as a reference, acknowledging the fact that the number of available samples declined over time due to an increasing number of weaned infants. We therefore chose to perform more robust analyses on infants who were weaned after month 7 (*n* = 20). At months 9 and 12, there were 9 B infants. Bacterial richness (months 3 and 5: $P < 0.005$; months 7, 9, and 12: $P < 0.05$) and Shannon effective diversity (months 5 and 7: $P < 0.005$; months 9 and 12: $P < 0.05$) were significantly higher in F infants than in B infants from month 3 on (**Figure 3**A). However, no significant difference was observed between F+ and F−. β-diversity analysis showed different phylogenetic makeup of the fecal microbiota between F and B infants at all time points measured (Figure 3B). The most dominant phylum in B infants was Actinobacteria, whereas the fecal microbiota of both F groups was dominated by Firmicutes, independent of bifidobacteria supplementation (Figure 3C). At the family level, bifidobacteria supplementation of the infant formula correlated with a significant reduction in the relative abundance of Bacteroidaceae at month 1 (Figure 3D). These differences largely disappeared at month 3, supporting the overall finding of the present study that bifidobacterial intervention does not have any long-term effects on fecal microbial communities. More detailed analysis at the phylotype level revealed additional differences between the F+ and F− microbiota over the 1-y intervention period. The F− group was associated with a significantly higher relative abundance of *Bacteroides fragilis* (OTU18; 2.2% ± 1.3%; $P < 0.005$)

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TABLE 1
Study population characteristics[1]

	F+ (n = 48)	F− (n = 49)	B (n = 9)
Maternal characteristics			
BMI before pregnancy, kg/m^2	23.6 ± 4.1	22.4 ± 4.1	23.3 ± 2.6
Age at birth, y	31.8 ± 4.7	32.4 ± 5	33.2 ± 2.7
Nullipara, *n* (%)	35 (73)	41 (84)	7 (78)
Birth characteristics			
Gestational age, d	279 ± 8	278 ± 8	281 ± 6
Weight, g	3399 ± 549	3191 ± 424	3515 ± 298
Size, cm	51 ± 3.2	51 ± 2.1	52 ± 1.7
Vaginal delivery, *n* (%)	28 (58)	27 (55)	3 (33)
Cesarean delivery, *n* (%)	20 (42)	22 (45)	6 (67)
Sex, *n* (%)			
Male	16 (33)	19 (39)	5 (56)
Female	32 (67)	30 (61)	4 (44)
Feeding characteristics			
Breastfed during first year, *n* (%)	37 (78)	38 (78)	9 (100)
Mean duration, mo	4.5 ± 3.6	5 ± 3.6	11.6 ± 1
Exclusively formula-fed, *n* (%)	11 (22)	11 (22)	0
Mean age at start of solid food intake, mo	5.2 ± 0.8	5 ± 0.8	5.7 ± 0.7
Probiotic supplementation,[2] *n* (%)	3 (6)	6 (12)	0
Infantile disease pattern, *n* (%)			
Fever	33 (69)	24 (49)	4 (44)
Virus with diarrhea	5 (10)	7 (14)	2 (22)
Diarrhea (nonviral)	8 (17)	8 (16)	2 (22)
Antibiotics	9 (19)	8 (16)	1 (11)
Drugs (except antibiotics)	28 (58)	17 (35)	1 (11)

[1] Values are means ± SDs unless otherwise indicated. There were no significant differences between the groups. B, exclusively breastfed; F+, intervention formula-fed; F−, placebo formula-fed.
[2] Bigaia (*L. reuteri*; Pädia) or Probiobact (8 *Lactobacillus* species; basic world).

and one *Blautia* species (OTU8; 3.0% ± 2.4%; $P < 0.005$) over the first year, while these species were low in F+ (0.04% ± 0%; 1.4% ± 1.3%) and B infants (0.3% ± 0.7%; 1.0% ± 1.4%). *B. longum* (OTU1) was characteristic of fecal microbiota in B infants compared with F infants throughout year 1 (37% ± 9.8% vs. 15.5% ± 4.8%; $P < 0.0005$), whereas one *Streptococcus* species (OTU9) was specific for both F groups at month 1 (11.9% ± 8.9% vs. 1.1% ± 1.3%; $P < 0.0005$) and *Ruminococcus gnavus* (OTU3) was higher in F infants than in B infants throughout year 1 (9.1% ± 4.5% vs. 3.5% ± 1.8%, $P < 0.05$). Analysis of the 10 most abundant OTUs in each F group revealed that a total of 5 OTUs belonged to the genus *Bifidobacterium*, including the 2 most dominant species (OTU1 and 2), which were shared between F+ and F− (**Supplemental Figure 4**). An EzTaxon sequence comparison showed that OTU1, OTU2, and OTU4 had a 100% sequence identity with the supplemented species *B. longum* subspecies *infantis*, *B. breve*, and *B. bifidum*, respectively, although identification at the species level is impossible due to similarity with other bifidobacteria (**Supplemental Table 2**).

Presence of fucosylated HMOs correlated with the occurrence of bifidobacteria

Breastfeeding was associated with high relative abundances of OTU1 (100% sequence identity to *B. longum*) (**Figure 4**A). The B group was also characterized by increased relative abundance of OTU3 (100% to *R. gnavus*) and OTU4 (100% to *B. bifidum*) when compared with the F groups. Proportions of OTU1 were significantly higher in B infants during year 1 (mean relative abundance of 37%). Analyses of maternal breast milk by HPLC-fluorescence revealed that 22 mothers were "secretors" and 5 mothers were "nonsecretors" (Figure 4B). "Secretor" mothers were characterized by high concentrations of the α1–2-fucosylated-HMO 2′fucosyllactose (Figure 4C). The prevalence of OTU1 was dependent on maternal secretor status. Of the infants born to "secretor" mothers, 50% were positive for OTU1, whereas none of the infants born to "nonsecretor" mothers (n = 5) was positive (Figure 4D).

Fecal metabolites discriminated between F+ and F− at an early age

Nontargeted metabolomics revealed that metabolite profiles were clearly distinct at neonatal age between B, F+, and F− infants, and they converged over time to reach profiles that were very different from the beginning of the study (**Figure 5**A). This deviation from neonatal profiles was mirrored by shifts in the microbiota over time (Figure 5B). Metabolite and microbiota data were then combined to search for differences at early (month 1), mid- (month 7), and late (month 12) points in the study. OPLS-DA was applied to determine differences between B, F+, and F− infants. We observed that fecal metabolites and OTUs discriminated between F+ and F− infants at the age of 1 mo (Figure 5C). At months 7 and 12, metabolites were different between B and F infants, but no longer between F+ and F−. Most of the metabolites discriminating between B, F+, and F− were assigned to sterol lipids, glycerophospholipids, and fatty acids.

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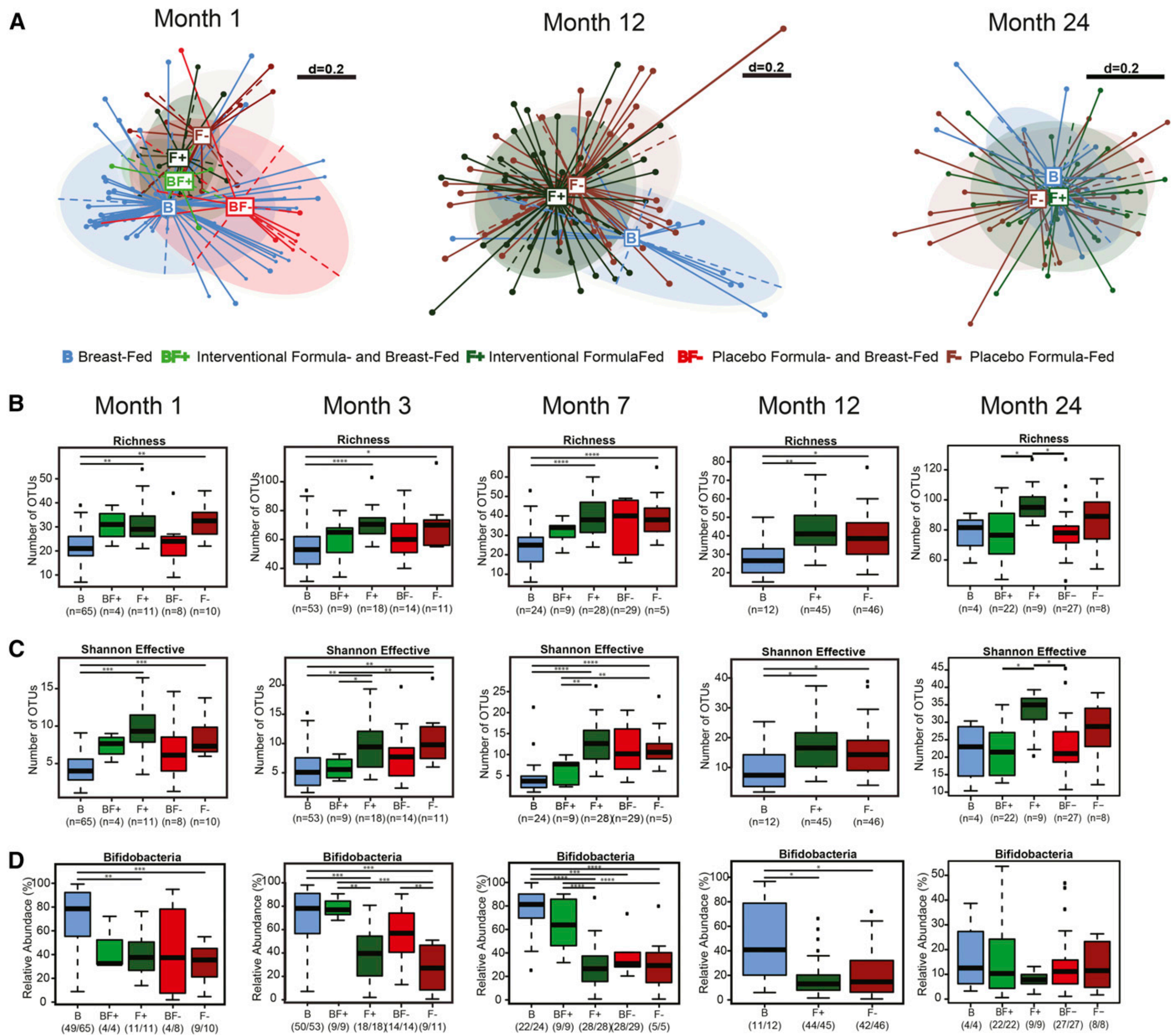

FIGURE 2 Differences in fecal microbiota in all infants according to feeding categories. β-Diversity analysis (A) is shown in nonmetric multidimensional scaling plots computed from generalized UniFrac distances, which were calculated from normalized OTU tables and phylogenetic distance trees. The different colors indicate feeding regimens at time of sampling. β-Diversity differed between B and F infants at month 1 ($P < 0.05$) and 12 ($P < 0.05$), with no long-term effect at 2 y of age ($P > 0.05$). Species richness (B) and Shannon effective counts (C) were calculated from normalized OTU tables with OTUs that had a minimum relative abundance of 0.5%. Cumulative relative abundance of OTUs (D) was determined with a minimum of 97% sequence identity to any member of the genus *Bifidobacterium*. *,**,***,****Significance between 2 values: $^*P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.0005$, $^{****}P < 0.00005$; the level of significance is indicated in the bar plots. Number of infants (n) is shown in parentheses below each box plot (also applies to the β-diversity data shown in panel A). B, exclusively breastfed; BF+, breast- and intervention formula-fed; BF−, breast- and placebo formula-fed; F, formula-fed; F+, interventional formula-fed; F−, placebo formula-fed; OTU, operational taxonomic unit.

Moreover, a correlation of metabolites and OTUs showed that 6 OTUs were involved in the feeding-specific shaping of the fecal ecosystem at month 1 and therefore contributed to the separation of the feeding groups (**Supplemental Table 3**). At month 7, no OTU- or metabolite-specific profile was observed anymore. Differences between breast and formula groups were maintained until the end of intervention at 1 y. Correlations of metabolites and OTUs at month 1 revealed a relation between F+-specific metabolites and 2 molecular species (OTU 4, *B. bifidum*; OTU142, *Lactococcus* species), while F−-specific metabolites showed a correlation with *Bacteroides* spp. (OTU10, OTU18) and *Odoribacter* species (OTU96). One of the F−-specific metabolites was assigned to LysoPE (15:0) and was highly correlated to OTU10 (*Bacteroides* species). Other detected F+- or F−-specific metabolites and their functions remain unknown.

None of the top 50 ranked metabolites associated with B infants at month 1 correlated with any OTU. Correlations between OTUs and lipids (fatty acids, sterol lipids, and glycerophospholipids), as well as unknown metabolites, were observed at month 7 (OTU1, *B. longum*) and month 12 (OTU14, *Enterococcus* species; OTU1 and OTU1653, both *Bifidobacterium* species; OTU108 and

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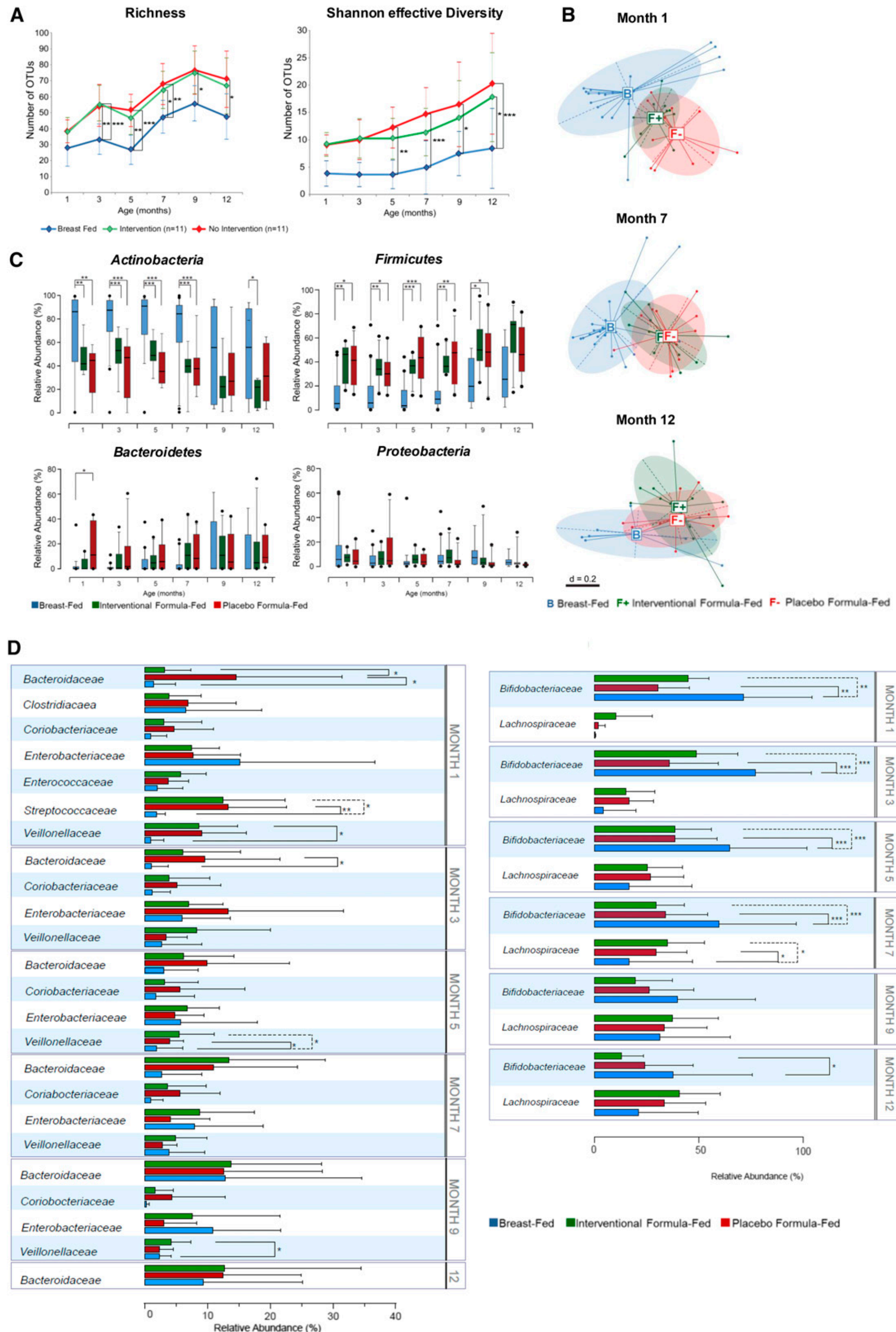

FIGURE 3 Microbiota changes throughout the first year of life in B, F+, and F− infants. Bacterial richness and Shannon effective counts from months 1 to 12 including infants from the F+ (n = 11), F− (n = 11), and B (months 1–7: n = 20, months 9 and 12: n = 9) infants are shown in panel A. Relative sequence abundance of major bacterial phyla is shown in panel B. An ANOVA framework was used with significance ($P < 0.05$) based on Benjamini-Hochberg corrected P values obtained by Wilcoxon's nonparametric test. β-Diversity analysis is shown in panel C in nonmetric multidimensional scaling plots computed from phylogenetic distances. Families with a minimum of 3% relative abundance in ≥1 of the 3 feeding groups are listed in panel D. Bifidobacteriaceae was the most dominant family from month 5 onward, followed by Lachnospiraceae and Bacteroidaceae, which both increased substantially from month 5 on. *,**,***Significance of OTUs or SCFAs to the corresponding feeding group (as indicated by the color of the stars): *$P < 0.05$, **$P < 0.005$, ***$P < 0.0005$. B, exclusively breastfed; F+, interventional formula-fed; F−, placebo formula-fed; OTU, operational taxonomic unit; SCFA, short-chain fatty acid.

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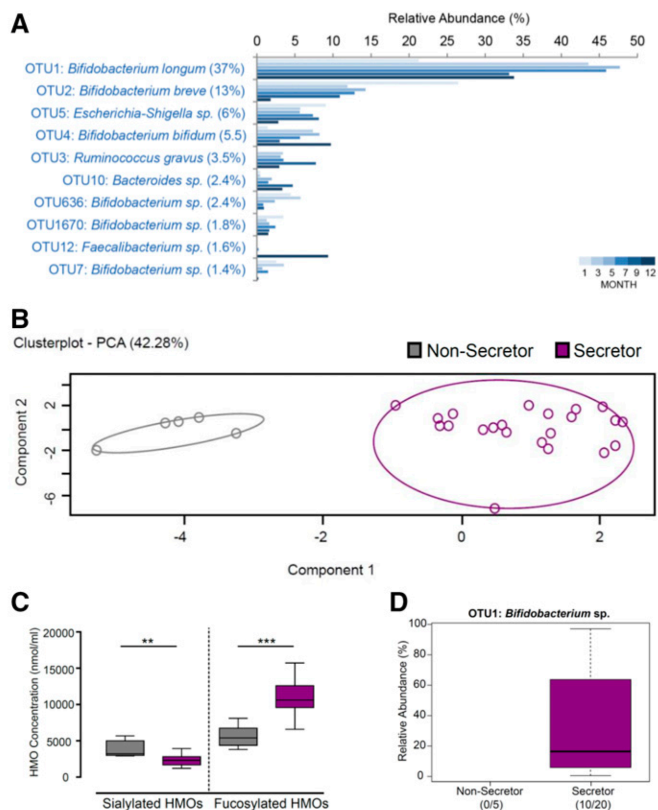

FIGURE 4 Impact of breastfeeding on the infant fecal microbiota. (A) OTU occurrences in B infants throughout the first year of life. Numbers in parentheses indicate mean relative abundance of the corresponding species in year 1, calculated from normalized OTU tables (months 1–7: $n = 20$, months 9 and 12: $n = 9$). (B) PCA of HMOs measured in 27 breast milk samples showing distinct clusters ($P < 0.05$) depending on the maternal secretor status (secretor mothers: $n = 22$, nonsecretor mother: $n = 5$). (C) The secretor status determined the concentrations of sialylated ($P < 0.005$) and fucosylated ($P < 0.0005$) HMOs. (D) OTU1 (identified as *Bifidobacterium longum*) was absent in feces of infants breastfed by nonsecreting mothers ($n = 5$) ($P > 0.05$). B, exclusively breastfed; HMO, human milk oligosacharride; OTU, operational taxonomic unit; PCA, principal component analysis.

OTU84, both *Lactobacillus* species). One of the main discriminating metabolites with a high rank after OPLS-DA analysis was exact *m/z* 552.3366, which was increased in the breastfed group at all measured time points and was assigned as a glycerophospholipid. In contrast, exact *m/z* 407.2455, assigned as a sterol lipid–like metabolite, was dominant in the feces of formula-fed infants through month 7.

We further assessed SCFA profiles in feces from the B and F infants using UHPLC-Q-ToF-MS (Figure 5D). The B group was associated with lower proportions of propionate, butyrate, valerate, and isovalerate, whereas pyruvic and lactic acid were detected at significantly higher concentrations. No significant differences in the SCFA profiles were detected between the F+ and F− groups. Independent of feeding group, the concentrations of propionic, butyric, isovaleric, and valeric acid increased over time.

Exogenous bifidobacteria failed to colonize the infant gut

Although of primary interest, the question of whether exogenous probiotic strains provided at early age are able to colonize the human intestine has not been addressed to date. Hence, strain-specific primers were designed to assess the presence of supplemented bifidobacteria in feces. Fecal samples collected from F+ infants at months 4, 12, and 24 were examined. As shown in **Figure 6**, *B. bifidum*, *B. breve*, and *B. longum* were detected in almost all infants at month 4, but in <50% of the infants at month 12. Detection limits ranged between 10^3 and 10^5 CFU/g feces (**Supplemental Figure 5**). This obvious washout of formula-derived bifidobacteria might have occurred due to a lower intake of formula at the end of the intervention or due to competitive exclusion by indigenous bifidobacteria. At month 24, none of the infants was positive for any of the strains. *B. longum* subspecies *infantis* could not be detected at any of the time points measured.

DISCUSSION

This placebo-controlled intervention study clearly demonstrated that bifidobacteria supplementation of infant formula does not substantially affect fecal microbial α- and β-diversity or proportions of bifidobacterial sequences during the first year of life. Such an intervention is therefore likely not to compensate for differences in microbiota composition observed between breast- and formula feeding. This is in contrast to some cultivation-based studies in which bifidobacteria intervention accounted for changes in early life fecal bacterial composition (11, 32). Although the F− and BF− groups significantly differed from the B, F+, and BF+ groups at month 1, these differences disappeared over time. In contrast to the use of synbiotic infant formula containing bovine milk–derived oligosaccharides and *B. animalis* subspecies *lactis* in a study from the literature (33), the supplementation with bifidobacteria alone was insufficient in changing the microbiota and metabolite profile over a longer period of time in our study. The responsiveness of the gut microbiota to the intervention during the first weeks of life was probably driven by the substantial microbial reorganization during this time that might have favored transient persistence of the exogeneous strains (34).

To confirm colonization of the supplemented bifidobacteria, we performed strain-specific analysis during and 1 y after the feeding period. We detected *B. bifidum*, *B. breve*, and *B. longum* in almost all samples during the intervention period, yet the frequency of detection was higher in month 4 than in month 12. At 2 y of age, the strains were no longer detectable, strongly suggesting that the supplemented bifidobacteria failed to stably colonize the infant gut due to competition within the ecosystem over time. Similar findings have been reported before (35). On the other hand, a recent study in adults confirmed the possibility that allochthonous bifidobacteria engraft in a "native" microbial ecosystem, likely depending on bacteria-related fitness factors or individual gut community structures (36). One possible approach to support the persistence of exogenous bifidobacteria is to add oligosaccharides that stimulate their growth in the infant gut (37).

While we detected 3 of the 4 strains in infant feces, *B. longum* subspecies *infantis* was not detectable. We checked the specificity of our assay by testing the type and other available strains of this species and found that primers were specific and that the strain was detectable in the supplemented formula. Viable cell numbers in the infant formula were determined by the provider via cultivation and confirmed to be $>10^7$ CFU/g. Possible explanations for the absence of *B. longum* subspecies *infantis* in

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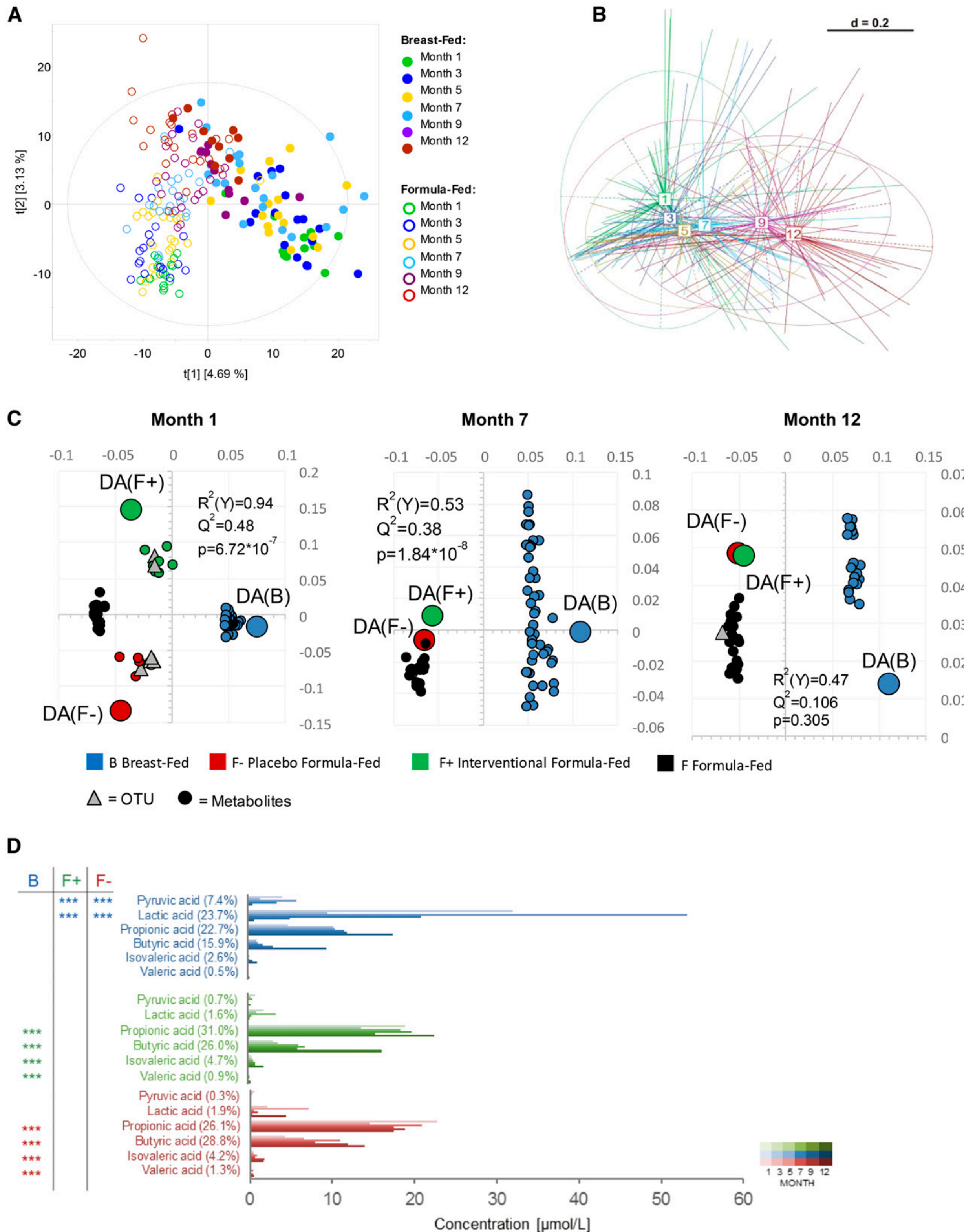

FIGURE 5 OTU and metabolite dynamics over time. Metabolite profiles (A) and microbiota development (B) were computed from generalized UniFrac distances that were calculated from normalized OTU tables and phylogenetic distance trees from months 1 to 12 in B and F infants (months 1–7: $n = 42$, months 9 and 12: $n = 31$). Loadings plots of main discriminating and correlating features between the feeding groups, based on microbiota and metabolite data (circles = metabolites, triangle = OTU), are shown in panel C. At month 1 the F+ and F− groups clustered apart ($P < 0.05$), but no discrimination between the groups was detected at later time points ($P < 0.05$). The list of discriminative features is provided in Supplemental Table 3. Fecal concentrations of SCFA in B (months 1 and 7: $n = 20$, month 12: $n = 9$) and F (months 1–12: $n = 11$ in either group) infants are shown in panel D. Kruskal-Wallis test followed by the Nemenyi significance test was applied for multiple comparisons of mean rank sums of each SCFA in the different groups. Colors for feeding groups: B (blue), F+ (green), F− (red); time points for months 1, 3, 5, 7, 9, and 12 are color-coded from light to dark. B, exclusively breastfed; DA, discriminant analysis; F, formula-fed; F+, interventional formula-fed; F−, placebo formula-fed; OTU, operational taxonomic unit; SCFA, short-chain fatty acid.

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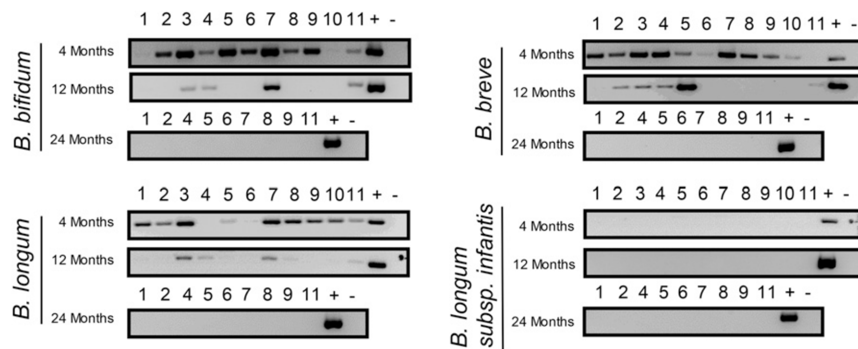

FIGURE 6 Strain-specific detection of the 4 supplemented bifidobacteria in fecal samples of 4-, 12-, and 24-mo-old infants exclusively fed the intervention formula. Primer sets were validated in silico with a Basic Local Alignment Search Tool search against the National Center for Biotechnology Information database and through polymerase chain reaction with DNA from type and additional available strains (Supplemental Figure 5). At months 4 and 12, all F+ infants (n = 11) were analyzed, but at month 24 we could follow up with only 9 of these infants. F+, intervention formula-fed.

the fecal samples tested include competitive exclusion of the strain in the intestine or loss of viability during postdelivery processing of the formula powder by the participants at home.

Antibiotic treatment or maternal programming might also prepare niches for the colonization of exogenous bacteria (38, 39). We will address the question whether allochthonous bifidobacteria better engraft in the infant gut in a follow-up study by using infant-derived isolates of dominant bifidobacteria, including strains with 16S rRNA gene sequences matching those of OTU 1 and 2.

In previous studies, breastfeeding was shown to be associated with increased relative abundances of Actinobacteria and Firmicutes at the expense of Proteobacteria (40). In addition, and also consistent with our findings, bacterial richness and diversity were lower in B infants than in F infants (41) and bifidobacteria dominated the gut microbial ecosystem of B infants at all times during year 1 (42). It is well accepted that HMOs specifically promote the growth of bifidobacteria (43). In our study, breastfeeding was associated with a higher relative abundance of 2 *Bifidobacterium* species and 1 *Ruminococcus* species compared with formula feeding. OTU1, classified as *B. longum*, was found to be by far the most dominant species found in B infants during the first year (mean relative abundance of 37%). Analyses of the breast milk showed that the prevalence of this species was dependent on the maternal secretor status. These results are consistent with published data demonstrating that a relative abundance of bifidobacteria correlates with the maternal secretor status, suggesting that an inactive allele of the maternal fucosyltransferase 2 (*FUT2*) gene (in "nonsecretor" mothers) leads to delayed and decreased colonization by bifidobacteria (44). We also showed that the presence of fucosylated HMOs favors the selection of specific bifidobacteria; however, differences between infant formula and breast milk were transient and community profiles converged after 2 y, supporting the view that the change from liquid to solid food is a major driver in the development of the complex gut microbial ecosystem (2, 45). In gnotobiotic mouse experiments, sialylated milk oligosaccharides have recently shown to change the microbiota and to promote growth in the context of infant undernutrition (46), suggesting a functional role of the developing infant microbiota on health conditions.

More specifically, we demonstrated that the supplementation of infant formula with bifidobacteria inversely correlated with *B. fragilis* colonization throughout the first year of life, showing similarities to the breastfed gut ecosystem, while placebo-fed infants were more often colonized by this species. *B. fragilis* contributes to the development and maturation of the infant immune system (47), but it also encodes virulent metalloproteases and other pathogenic features, making it one of the most popular anaerobic pathogens of the human gut (48). Moreover, one recent study showed that *Bacteroides* species are less abundant in Russian infants but are dominant in Finnish and Estonian infant cohorts. However, it remains to be seen whether increased *Bacteroides* abundance in the latter 2 populations contributes to a higher risk for the early onset of autoimmune diseases (49).

A specific feature of the present study is the implementation of high-resolution metabolome analysis to characterize the luminal milieu. We demonstrated that the metabolite profile in the intervention group significantly differed from the one observed in the placebo formula group and the solely breastfed group at the neonatal stage. Further, metabolites differed between formula- and breastfed groups throughout the first year. Lipids and unknown metabolites primarily accounted for these differences. It is well accepted that the metabolite profile of the gut lumen is dependent on the structure and function of the resident microbiota (50, 51). One recent study evaluated the use of probiotics in preterm infants and their impact on the microbiome and metabolome and concluded that metabolite profiles are different between probiotic and control groups, which strengthens our results on the discrimination of B and F infants over time (52). Although little insight is available for nontargeted metabolomics of healthy B and F infants, Wang et al. (53) identified 15-methylhexadecanoic acid, galactitol, and maltose as discriminating metabolites for breastfeeding, and β-alanine, dodecanoic acid, glycolic acid, decanoic acid, and tyramine for formula feeding. In contrast, we identified dodecanoic acid to be associated with breastfeeding at month 1, clearly demonstrating the need to perform additional validation studies. It should be pointed out that the fecal metabolome is a complex matrix (54) and the classification and identification of "unknown" metabolites is challenging. Several databases offer an overview of metabolite compositions (54, 55). Nevertheless, the vast majority of them (80%) still remain unknown, and a conclusion related to health or disease susceptibility is not possible at this stage.

In conclusion, this placebo-controlled intervention study showed that bifidobacteria-supplemented formula modulates the

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infant microbiome at very early stages in life with no detectable long-term consequences for gut microbiota assembly or function. The impact of sequentially changing bacterial and metabolite profiles on human health is unclear and requires additional research. One additional hallmark of this study is the observation that bifidobacteria ingested from formula were washed out of the infant gut over time and failed to persistently colonize beyond intervention despite the high prevalence of other bifidobacteria in all feeding groups even after 2 y of age.

We thank Thomas Skurk from the Human Study Core Facility of the ZIEL Institute of Food & Health at Technical University of Munich for medical assistance, and Caroline Ziegler and Sandra Fischer from the ZIEL Core Facility Microbiome/NGS for outstanding support with sample processing and statistical analysis.

The authors' responsibilities were as follows—DH: developed the hypothesis and initialized the intervention study; MB, TC, and DH: designed the experiments and wrote the manuscript; MB, TC, IL, JW, and DH: coordinated and supervised the data analysis on microbiota profiling; TVM, ML, and PS-K: coordinated and supervised the data analysis on metabolite profiling; CA and LB: coordinated and supervised the data analysis on HMO profiling; MB, TVM, ML, MXM-G, and CA: performed the experiments; and all authors: provided feedback on the manuscript. None of the authors reported a conflict of interest related to the study. Töpfer GmbH did not influence any portion of this study, including study design, contact with participants, sample collection and analysis, data management, interpretation of the results, and writing of the manuscript.

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