Early life gut microbiota profiles linked to synbiotic formula effects: a randomized clinical trial in European infants

Ilias Lagkouvardos, Evangelia Intze, Monika Schaubeck, James Pk. Rooney, Christina Hecht, Hugues Piloquet, Thomas Clavel

The American Journal of Clinical Nutrition 117 (2023) 326–339

The American Journal of CLINICAL NUTRITION

journal homepage: www.journals.elsevier.com/the-american-journal-of-clinical-nutrition

Original Research Article

Early life gut microbiota profiles linked to synbiotic formula effects: a randomized clinical trial in European infants

Ilias Lagkouvardos [1,2], Evangelia Intze [2], Monika Schaubeck [3], James PK. Rooney [4], Christina Hecht [3], Hugues Piloquet [5], Thomas Clavel [1,*]

[1] *Functional Microbiome Research Group, Institute of Medical Microbiology, RWTH University Hospital, Aachen, Germany;* [2] *Institute of Marine Biology, Biotechnology and Aquaculture, Hellenic Centre for Marine Research, Heraklion, Greece;* [3] *HiPP GmbH and Co. Vertrieb KG, Pfaffenhofen, Germany;* [4] *Staburo GmbH, Munich, Germany;* [5] *Chronic Childhood Diseases Unit, Pediatric Department, Nantes University Hospital, Nantes, France*

A B S T R A C T

Background: Microbial colonization of the gastrointestinal tract after birth is an essential event that influences infant health with life-long consequences. Therefore, it is important to investigate strategies to positively modulate colonization in early life.

Objectives: This randomized, controlled intervention study included 540 infants to investigate the effects of a synbiotic intervention formula (IF) containing *Limosilactobacillus fermentum* CECT5716 and galacto-oligosaccharides on the fecal microbiome.

Methods: The fecal microbiota from infants was analyzed by 16S rRNA amplicon sequencing at 4, 12, and 24 months of age. Metabolites (e.g., short-chain fatty acids) and other milieu parameters (e.g., pH, humidity, and IgA) were also measured in stool samples.

Results: Microbiota profiles changed with age, with major differences in diversity and composition. Significant effects of the synbiotic IF compared with control formula (CF) were visible at month 4, including higher occurrence of *Bifidobacterium* spp. and *Lactobacillaceae* and lower occurrence of *Blautia* spp., as well as *Ruminoccocus gnavus* and relatives. This was accompanied by lower fecal pH and concentrations of butyrate. After de novo clustering at 4 months of age, overall phylogenetic profiles of the infants receiving IF were closer to reference profiles of those fed with human milk than infants fed CF. The changes owing to IF were associated with fecal microbiota states characterized by lower occurrence of *Bacteroides* compared with higher levels of Firmicutes (valid name Bacillota), Proteobacteria (valid name Pseudomonadota), and *Bifidobacterium* at 4 months of age. These microbiota states were linked to higher prevalence of infants born by Cesarean section.

Conclusions: The synbiotic intervention influenced fecal microbiota and milieu parameters at an early age depending on the overall microbiota profiles of the infants, sharing a few similarities with breastfed infants.

This trial was registered at clinicaltrials.gov as NCT02221687.

Keywords: infant gut microbiome, synbiotic, *Limosilactobacillus fermentum*, GOS, Cesarean section

Introduction

Infants are colonized by microbiota during birth [1, 2], and the first months of life are extremely important for the establishment of the gut microbiota and maturation of the immune system [3]. Although improvement of hygienic conditions reduces infant mortality due to infections, environments with low microbial biomass counteract natural colonization by commensal microbes. For example, infants living in rural compared with urban environments are exposed to a wider range of microorganisms and their products and are at lower risk of developing allergic diseases [4–6]. Additionally, birth via Cesarean section (CS) modulates the diversity and composition of the gut microbiota during the first months of life [7]. Hence, it is important to study processes influencing early microbial colonization as this is a critical time window during infant immune development [2].

Human milk (HM) is the gold standard for infant nutrition, influencing microbial communities owing to its prebiotic and probiotic components. It contains oligosaccharides that can be used as substrates by gut bacteria, especially bifidobacteria [8–10]. Furthermore, HM contains bacteria presumably important in seeding the infant's intestine [11, 12].

If breastfeeding is not possible, infant formula should support similar development of the intestinal ecosystem. Adding pre and/or

Abbreviations used: CF, control formula; CS, cesarean section; GOS, galacto-oligosaccharides; HM, human milk; IF, intervention formula; M4, month 4; sOTU, operational taxonomic unit (level of molecular species); ZOTU, zero-radius OTU (level of molecular strains).

* Corresponding author.
E-mail address: tclavel@ukaachen.de (T. Clavel).

https://doi.org/10.1016/j.ajcnut.2022.11.012
Received 22 April 2022; Received in revised form 5 November 2022; Accepted 17 November 2022
Available online 21 December 2022

probiotics to infant formula is one possible approach to support the development of the infant microbiota by nutrition [13].

Interventions with exogenous microbes has gained attention in modulating the infant gut microbiota. Small intervention studies showed moderate success in beneficially shaping the microbiota of infants born through CS using maternal vaginal fluid or fecal microbiota [14, 15]. However, interventions using microbiota transplantations are difficult to perform at larger scales due to complicated logistics (donor sample collection, screening, and preparation) and potential risks associated with applying undefined communities of microbes. Thus, well-defined probiotic microbes represent a sound intervention strategy to modulate the gut microbiota during infancy.

Lactobacilli are an important group of bacteria often used as probiotics and have been taxonomically reclassified recently [16]. However, their occurrence in the intestine of adults in industrialized countries is low, aligning with the concept of a decreasing diversity within gut microbiomes [17], likely because of unfavorable dietary habits and medication [17, 18]. Lactobacilli may thus be interesting for dietary intervention at an early age.

HM contains microorganisms that may contribute to some of the beneficial effects of breastfeeding. Hence, supplementing infant formula with bacteria from HM is a sound approach to positively modulate colonization in infants who cannot be breastfed [13]. *Limosilactobacillus fermentum* CECT5716 is one such HM-derived isolate for which probiotic properties have been shown [19, 20]. The safety of this strain was assessed in 3 published infant cohorts based on normal weight development, growth, and head circumference, along with the absence of adverse events [21–24]. Its efficacy was first reported by Maldonado et al. [22], who observed a reduction in gastrointestinal and respiratory tract infections upon application of an infant formula with galacto-oligosaccharides (GOS). GOS are carbohydrate polymers reported to support the growth of bifidobacteria [25, 26] and contribute to stool softness [27, 28]. One possible explanation for these protective effects might be the beneficial impact on the intestinal microbiome by the synbiotic combination. Previous qPCR analyses indicated increased counts of lactobacilli and bifidobacteria in infant stool after intervention [22], but detailed effects on gut microbial communities have not yet been investigated in a comprehensive manner.

The aim of the present study was to assess the effects of a synbiotic intervention formula (IF) with *L. fermentum* CECT5716 and GOS on fecal microbiota using 16S rRNA gene amplicon sequencing and by measuring milieu parameters, including pH, fecal water, IgA, calprotectin, and SCFAs. Therefore, a multicenter, randomized, controlled, double-blind intervention trial was conducted, enrolling 540 French and Belgian infants, including measurements at the age of 4, 12, and 24 months.

Methods

Study product

The synbiotic IF was a standard infant formula enriched with prebiotic GOS (0.02 g/g) and the probiotic strain *L. fermentum* CECT5716 (at least 1.0×10^6 cfu/g). The follow-on formula was a standard follow-on formula enriched with 0.03 g/g GOS and 1.5×10^6 cfu/g. The control formula (CF) was similar in all components but did not contain pre and probiotics. All study products were in accordance with EC Directive 2006/141/EC. Formula consumption was ad libitum, starting within the first 3 d after inclusion (1 mo ± 7 d) until 6 months of age, followed by follow-on formula until 12 months of age. At 4 mo, the approximate drinking volume in both formula groups was about 778 mL.

Study design

Healthy infants ($n = 540$) were included in a double-blinded controlled trial at 1 of 40 French and 1 Belgian site between August 2014 and May 2018. Infants whose parents had chosen not to breastfeed or were not able to breastfeed prior to study inclusion were allocated randomly to 1 of 2 formula groups ($n = 230$ CF, $n = 230$ IF). The infants in the breastfed reference group ($n = 80$) were mainly fed HM, defined as a maximum of 1 formula meal per 24 h and willingness of the mother to continue breastfeeding until at least the age of 4 mo. Power calculation was based on the rate of gastrointestinal infections during the first year of life (results on this primary outcome will be described elsewhere). Dynamic randomization was used to minimize the imbalance between the 2 formula groups taking strata defined by the combination of the following 2 factors: mode of birth (vaginal or CS) and presence of a history of breastfeeding at inclusion (yes or no). For inclusion in the formula groups, infants had to be exclusively formula-fed at the time of randomization. Exclusion criteria included the following: history of neonatal health problems; clinical evidence of chronic illnesses or gastrointestinal disorders; diagnosed metabolic or immune disorder; consumption of formula for special medical purposes; oral antibiotic treatment at the time of enrollment. Concomitant antibiotic treatment during the study was recorded and was not significantly different between formula groups. This clinical trial was prospectively registered in clinicaltrials.gov under the name "The Combiotic-Study (GOLFIII)" (NCT02221687) and carried out in accordance with the Declaration of Helsinki and the Good Clinical Practice standards as far as they apply to nutritional trials. Ethical approval for the study was obtained from the Ethics Committees Ouest IV of Nantes, France, and University Hospital Saint-Luc, Belgium (Nr. 15/14 from April 1, 2014). Parents of all included infants provided written informed consent for study participation. The CONSORT flow chart is shown in Supplemental Figure 1. The characteristics of the full analysis set population are shown in Supplemental Table 1.

Sample collection

Stool samples were collected by the parents directly from diapers as soon as possible after defecation at 4, 12, and 24 months of age. Stool from morning diapers was not collected due to potentially long delay after defecation. Parents were provided spatulas and collection jars and were advised to fill the jars with a minimum quantity of stool while avoiding contact with urine and other liquids. Jars with stool samples were placed in airtight bags and refrigerated at +2°C to +8°C for a maximum of 24 h before parents handed them to a local laboratory.

Upon arrival at the local laboratories, stool samples were transferred into 2 microtubes, 1 for qPCR and microbiota analysis by sequencing, and 1 for measurement of fecal milieu parameters (calprotectin, secretory IgA, pH, fecal water, and SCFAs). Microtubes were stored at −20°C until shipment to the major laboratory. Samples were then further stored −20°C until analysis.

Fecal milieu parameters

The pH of the stool samples was measured with a pH meter (pH 1000 L, VWR International). Prior to SCFA analysis, the humidity rate of the stool samples was determined using an oven (Memmert GmbH + Co.KG) at 105 ± 5°C for 24 h. Weight difference before and after drying due to water loss was assessed, and the humidity rate was calculated in percentage afterward. To measure SCFAs, stool samples were homogenized, dissolved in water, and ultrasonicated before centrifugation. SCFAs were extracted with ethyl ether after adding

hydrochloric acid solution and the internal standard. A fraction of the organic phase was dried with sodium sulfate and introduced into a vial for gas chromatography. The samples and the calibration range were analyzed by gas chromatography with flame ionization detection (Shimadzu GC-2010 Plus) using a 30 m × 0.25 mm × 0.25 μm J&W Scientific capillary column. The obtained SCFA results were expressed as mg/g dry fecal matter. Secretory IgA (sIgA) concentration was determined by ELISA (ImmuChrom ELISA Kit, Immuchrom GmbH) according to the manufacturer's instructions. Calprotectin concentration was measured by ELISA or turbidimetric assay (Bühlmann fCAL tests) according to the manufacturer's instructions.

Stool DNA extraction

DNA was extracted from stool samples using the Maxwell 16 Tissue DNA Purification kit (Promega) following a protocol with dual cell lysis (mechanical and chemical), which is as follows: 200 mg of stool in lysis solution was disrupted for 30 s using 0.1 mm zirconium beads (VWR International) and a FastPrep homogenizer (MP Biomedicals, Valiant Co.). DNA isolation was carried out with a Maxwell MDx automated station (Promega) according to the manufacturer's instructions. DNA was quantified using a Qubit 2.0 fluorometer (Thermo Fisher Scientific).

Real-time PCR analyses

Real-time PCR was performed on an ABI 7500 Real-time PCR System (Applied Biosystems, Thermo Fisher Scientific). The first step consisted of checking the absence of PCR inhibitors in all the samples using the TaqMan Exogenous Internal Positive Control Reagent (Applied Biosystems, Thermo Fisher Scientific).

Each sample was then analyzed in duplicate for quantification of *Clostridioides difficile* and *Lactobacillaceae* spp. using Genesig kits (Primerdesign Ltd.) following the manufacturer's instructions. *Bifidobacterium* spp., *Limosilactobacillus fermentum*, and *L. fermentum* CECT5716 were quantified using internal methods with fluorogenic probes and Premix Ex Taq (Takara Bio Europe) [26]. *Enterobacteriaceae* members were quantified with DNA-intercalator TB Green Premix Ex Taq (Takara Bio Europe). All the results were expressed as gene copies per 200 mg of fecal sample.

16S rRNA gene amplicon library preparation and sequencing

The V3-V4 regions of the genes encoding 16S rRNA were amplified by PCR using primers 341F and 785R in a 25-μL reaction volume containing 12.5 ng of sample DNA [29]. PCR cycling was performed with an initial denaturation step at 95°C for 5 min, followed by 25 cycles at 98°C for 30 s, annealing at 55°C for 30 s, elongation at 72°C for 30 s, and a final extension at 72°C for 5 min. A 16S rRNA gene amplicon library was generated for each sample by adding dual indices and Illumina sequencing adapters using the Nextera XT Index kit. Each library was cleaned up with magnetic AMPure XP beads (Beckman Coulter), and the size was verified for at least 10% of the samples by capillary electrophoresis with a 2100 Bioanalyzer (Agilent Technologies). After quantification by fluorimetry (Qubit 2.0 Fluorometer), libraries were normalized to 4 nM and pooled before denaturation and sequencing (paired-end, 2 × 250, v2 chemistry) using an Illumina MiSeq.

16S rRNA gene amplicon data processing

The raw fastq files were processed at the level of molecular strains (ZOTUs) using the UNOISE3 method [30] from USEARCH11 (64-bit version) [31] as implemented in IMNGS [32] with settings of side trimming of 10 bases, amplicon size minimum >300 and maximum <500, minimum ASV (amplicon sequence variant) size = 4, trim score = 20, and other default settings. Denoised sequences were aligned and taxonomically classified with SINA [33] using SILVA release 138 as reference [34]. The taxonomic classification was further manually refined for sequences missing family and higher classification using EzBioCloud [35]. For analysis at the level of operational taxonomic units (molecular species; sOTUs), the denoised sequences (ZOTUs) from the previous step were clustered at 97% sequence similarity using USEARCH11. The aligned sequences of both ZOTUs and sOTUs were used to determine phylogenetic trees with the Neighbor-Joining method in MEGAX [36]. To identify the ZOTUs corresponding to the probiotic used for the intervention, all sequences were BLASTed against the reference sequence of *L. fermentum* CECT5716. ZOTU83 returned 100% sequence identity and formed a monophyletic cluster with 5 other molecular strains (Zotu404, Zotu288, Zotu672, Zotu514, and Zotu186) that all shared >98% sequence identity and were thus considered to represent the target. The contingency table summarizing the occurrence of molecular species (i.e., counts of quality-checked and assembled sequences), their taxonomy, and associated metadata about the samples are provided in Supplemental Table 2.

Downstream sequence data analyses

Processed 16S rRNA gene amplicon data was analyzed further in R using Rhea (v1.1.5) [37]. The first step of data analysis was normalization, either by division to minimal sampling size for cases where counts were required (e.g., β-diversity) or relative to 100 for statistics on taxa composition. Diversity within samples (α-diversity) was measured as richness (count of sOTUs) and its variant effective richness (i.e., count of species above 0.25% relative abundance independent of sequencing depth) [38]. Diversity between samples (β-diversity) was calculated using the generalized UniFrac method (α = 0.5) [39]. Ensuing dissimilarity matrices were visualized as either multidimensional scaling (MDS) plots or hierarchical clustering trees. Decomposition of groups of microbial profiles to reveal their intrinsic organization and the calculation of their β-diversity distances to reference points were performed with DivCom [40]. De novo clustering of microbial profiles was performed using Partitioning Around Medoids based on Calinski-Harabasz indices for selection of the optimal number of clusters (k).

Statistical analysis

Statistical analyses of microbiota data and gut milieu parameters were conducted as described in Rhea (v1.1.5) [37]. Differences in β-diversity were tested using permutational multivariate analysis of variance within the R package "adonis". Differences in gut milieu parameters and the relative abundance of taxa between groups were tested using the Kruskal-Wallis rank sum test, followed by pairwise comparisons using the Wilcoxon rank sum test. Differences in prevalence (number of positive samples) were evaluated using Fisher's exact test. Prior to statistical testing of microbial taxa, thresholds of prevalence and relative abundances were applied to reduce the number of tests on underpopulated and lowly abundant taxa. These thresholds were as follows: prevalence >30% within at least one group (e.g., one-third of infants positive for the given taxon with any feeding group) and median relative abundance >1% in at least one group. Correction to the Kruskal-Wallis test and Fisher's exact test was applied for the number of tests for each independent variable. For example, the effect of the parameter "Diet" across *N* taxa was tested with the Kruskal-Wallis test for relative abundance and Fisher's exact test for prevalence to compare the groups. Then, the corresponding *P*

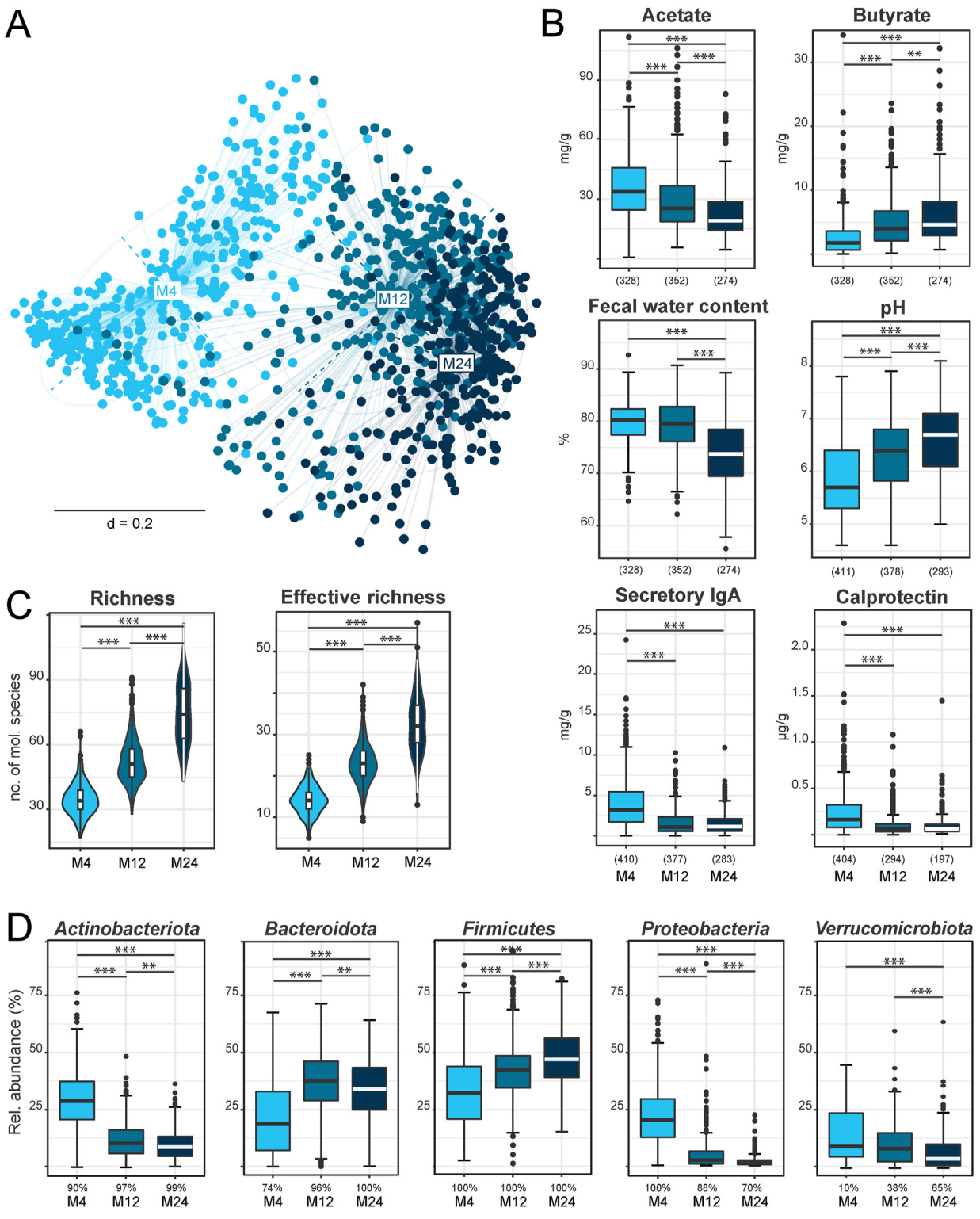
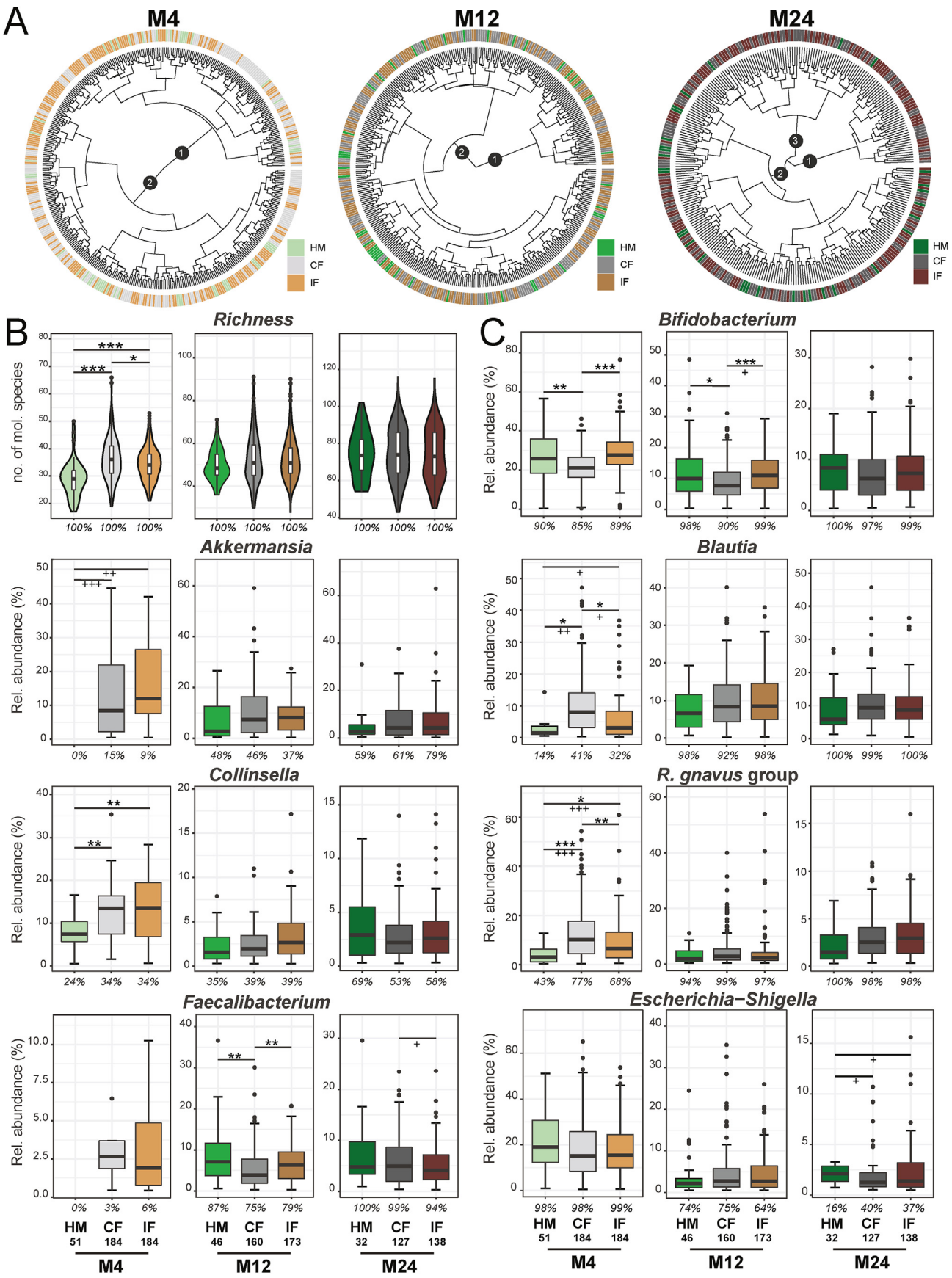


FIGURE 1. Fecal microbiota analysis of the infants over the first 2 years of life. (A) ß-Diversity analysis. Multidimensional (MDS) plot showing the similarity of individual microbial profiles (dots) from the infants at 4, 12, and 24 months of age (M4, M12, and M24, respectively). (B) Over time differences in fecal physicochemical parameters. The numbers in parentheses below the *x*-axis correspond to the number of values available for the corresponding variable (i.e., data were not available for the remaining samples). (C) α-Diversity is shown as richness (number of observed molecular species) and effective richness (species occurring in at least 1 sample at a relative abundance ≥0.25%). (D) Boxplots showing the relative abundance of the 5 bacterial phyla detected in the infant fecal samples. The percentages below the *x*-axis correspond to the fraction of samples positive for the given phylum, referred to as "prevalence." The total numbers of infants per age category are M4, $n = 419$; M12, $n = 379$; M24, $n = 297$. Statistics: adjusted *P* value *** <0.001, ** <0.01, * <0.05; Wilcoxon rank sum test. IgA, immunoglobulin A.



(caption on next page)

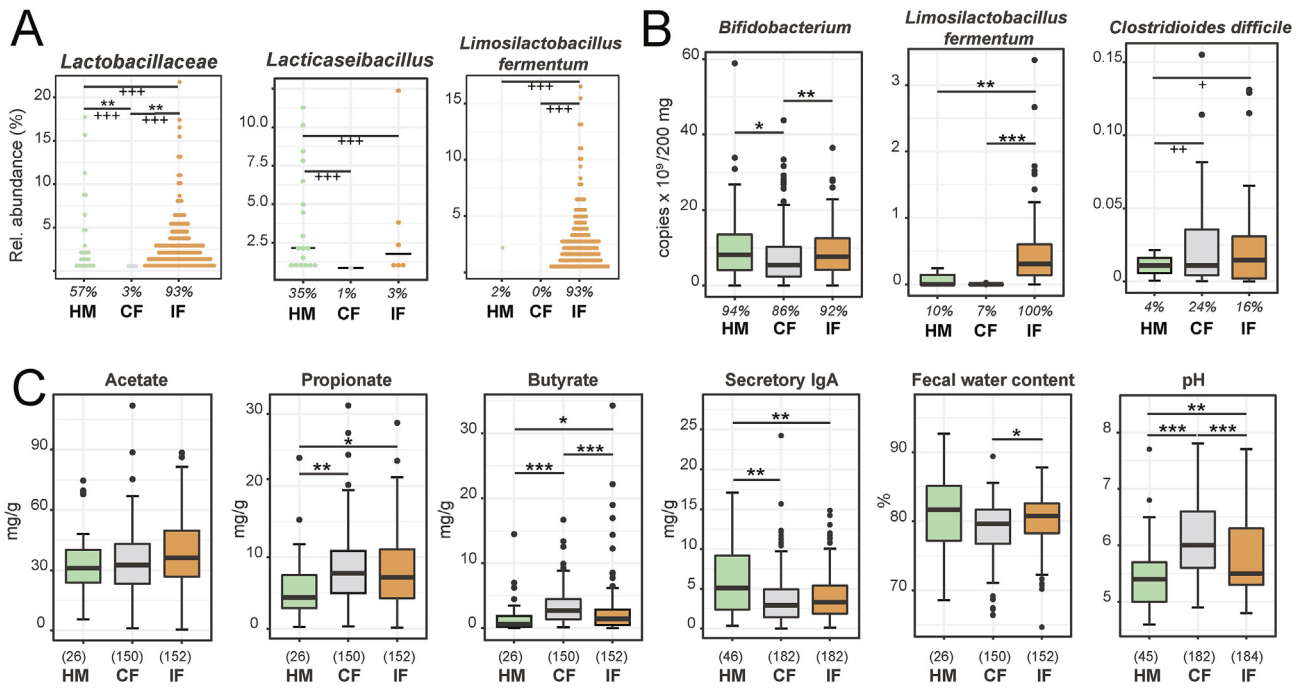


FIGURE 3. Effects of the synbiotic intervention on targeted microbiota composition data and fecal physicochemical parameters at 4 months of age. (A) Occurrence of lactobacilli in the 3 feeding groups based on amplicon sequencing data. Percentages below the respective *x*-axis indicate the prevalence, i.e., a fraction of samples positive for the given taxon. (B) Targeted quantification of specific bacteria by qPCR. (C) Differences in fecal milieu parameters between the feeding groups. The numbers in parentheses below the *x*-axis correspond to the number of values available for the corresponding variable. The total number of infants in each feeding group: HM, $n = 51$; CF, $n = 184$; IF, $n = 184$. Statistics: Stars (above the black lines linking the 2 groups with a significant difference) indicate differences in relative abundance using the Wilcoxon rank sum test. Crosses (below the lines) indicate differences in prevalence, i.e., the percentage of samples positive for the given taxon, as indicated below the respective *x*-axis and tested using Fisher's exact test. *, adjusted *P* value <0.05; **, <0.01; ***, <0.001. CF, control formula; HM; human milk; IF, intervention formula; IgA, immunoglobulin A; pH; qPCR, polymerase chain reaction.

values were corrected according to *N* repetitions. Whenever significance occurred after correction, pairwise Wilcoxon rank sum and/or Fisher's exact tests were applied, and the results were independently corrected. Benjamini-Hochberg method was applied in all cases for correction to account for multiple testing. Adjusted *P* values ≤0.05 were considered statistically significant. The group of breastfed infants, which was not blinded and randomized, was used as the reference for assessing the effects of the formulae.

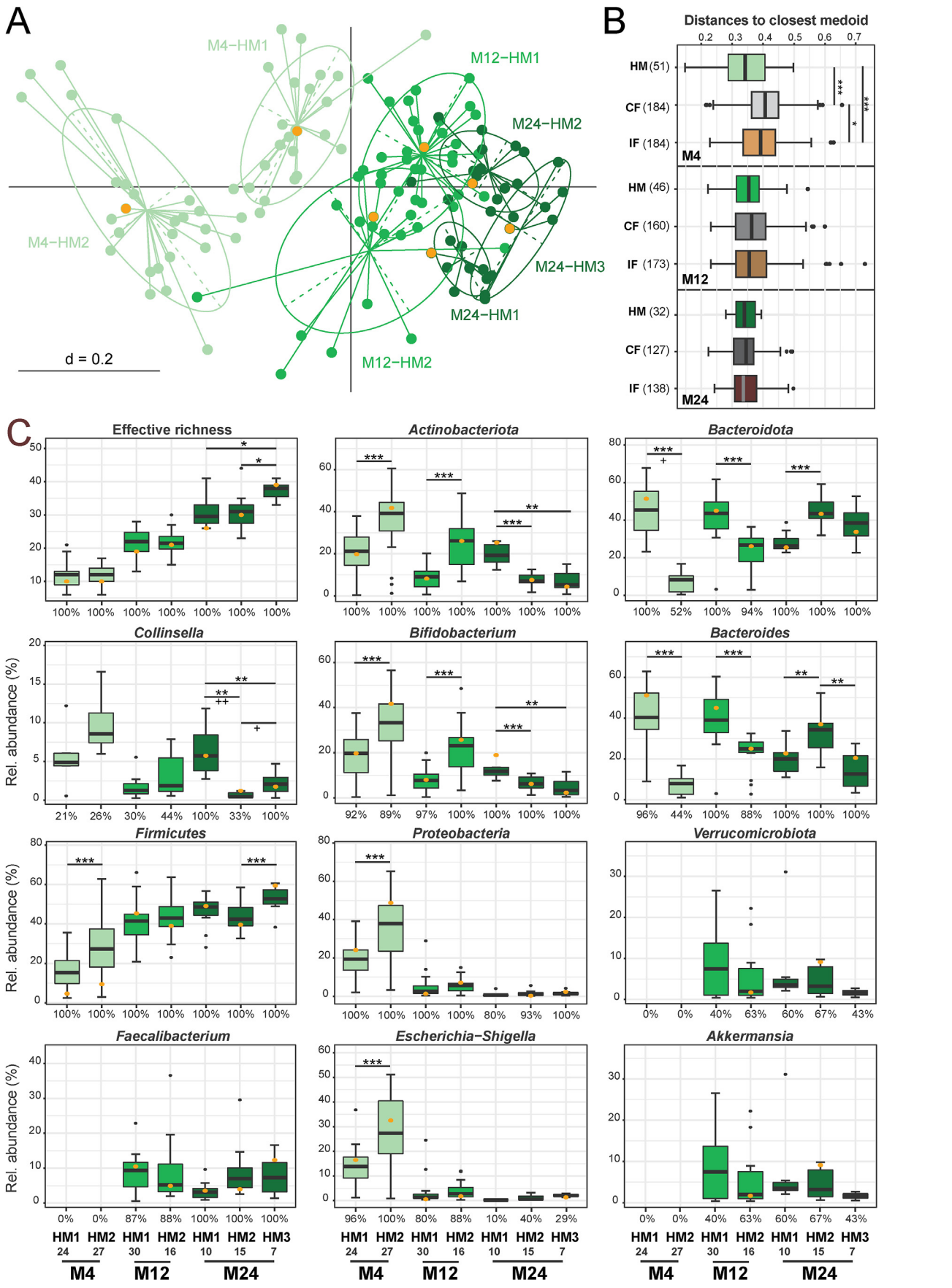
Besides the aforementioned analyses performed in Rhea, the effects of intervention groups and visit time points were also analyzed using generalized linear models using the lme4 package in R. In these analyses, random slopes were not included as there were not enough time points for all individuals to support such a fit. Furthermore, random effects of study center were not considered as a number of centers had low numbers of participants (i.e., <5), and therefore random effects by center were unlikely to be well estimated. To make postfit estimations, the R package emmeans was used to estimate marginal means or least-squares means, predicted from model fit for the subgroupings as required (i.e., across visit times or intervention groups). The R scripts used to generate this data are available in the supplemental information.

Results

Development of the intestinal ecosystem over time

After raw data processing, the final dataset contained ~111 million high-quality, assembled sequences (84,650 ± 20,438 per sample), representing a total of 319 sOTUs (66 ± 21 per sample, with a length of 432 ± 11 bases), which was used as a foundation for all other analyses. As observed in other cohorts [7, 39], phylogenetic profiles of the fecal microbiota in all infants (HM, IF, and CF) depended on age (month 4, M4; month 12, M12; and month 24, M24) (Figure 1A and Supplemental Figure 2A). Of note, the microbiota profiles were not affected by sample origin, i.e., the study center (data not shown). The specific microbial features in the feces of the infants at 4 months of age were accompanied by significantly higher concentrations of acetate, secretory IgA, and calprotectin; higher fecal water content as a marker for the softness of the stool; and lower pH and butyrate concentrations

FIGURE 2. Global effects of the synbiotic formula intervention on the infant gut microbiota. (A) Cladograms of inter-sample dissimilarities based on generalized UniFrac distances for each time point separately (M4, month 4 of age; M12, month 12; M24, month 24). Colors surrounding the branches indicate the feeding group: HM, human milk (green); CF, control formula (gray); IF: intervention formula (gold to brown). (B-C) Significant differences in richness and dominant bacterial genera between the feeding groups (B, main differences between HM compared to other 2 groups; C, IF to other to groups. Statistics: Stars (above the black lines linking the 2 groups with a significant difference) indicate differences in relative abundance using the Wilcoxon rank sum test. Crosses (below the lines) indicate differences in prevalence, i.e., the percentage of samples positive for the given taxon, as indicated below the respective *x*-axis and tested using Fisher's exact test. *, adjusted *P* value <0.05; **, <0.01; *** <0.001. The total numbers of infants per feeding group are indicated in bold letters at the bottom of the graphs. The HM group is a non-randomized reference group. CF, control formula; HM; human milk; IF, intervention formula; *R. gnavus, Ruminoccocus gnavus.*



(caption on next page)

compared with those at later time points (Figure 1B). The early time point (M4) was characterized by a lower complexity of bacterial communities (α-diversity) accompanied by high relative abundances of *Bifidobacterium* spp. (phylum Actinobacteriota; valid name Actinomycetota; formerly Actinobacteria) and *Enterobacteriaceae* (phylum Proteobacteria; valid name Pseudomonadota) in nearly all infants (Figure 1C and D and Supplemental Figure 2B). The relative abundance of these taxa decreased over time, which was paralleled by a steady increase in α-diversity, moving from an average of 35 (M4) to 75 (M24) observed molecular species (richness) and higher relative abundances of Bacteroidota (valid name; formerly Bacteroidetes) and Firmicutes (valid name Bacillota) with increasing age. The data presented here were confirmed by using a generalized linear mixed model with a random intercept for individual subjects and fixed effects for visit (4, 12, or 24 mo), study center, and delivery mode (Cesarean or vaginal delivery) (Supplemental Table 3a–b).

Synbiotic intervention modulated the fecal ecosystem early in life

Due to the observed age-dependent differences in fecal microbial profiles described above, the effects of the synbiotic intervention were investigated for each time point. Microbiota structure visualized as cladograms of phylogenetic distances showed deep branching of 2 to 3 clusters per time point that did not mirror feeding groups, i.e., infants in each group were scattered around the respective cladograms (Figure 2A). When compared with reference microbiota profiles of infants fed HM at M4, both the CF and IF groups were characterized by a more diverse ecosystem and a higher prevalence and relative abundance of strict anaerobes belonging to the bacterial genera *Akkermansia*, *Collinsella*, and *Faecalibacterium* (Figure 2B). When comparing the 2 formula groups, IF-fed infants were characterized by significantly higher relative abundances of *Bifidobacterium* spp. at M4 and M12. These bifidobacteria levels in IF-fed infants were comparable to those in infants from the HM reference group (Figure 2C). In contrast, relative abundances of *Blautia* spp., as well as *R. gnavus* and relatives, were lower in the IF group. These differences observed at M4 disappeared over time, except for a significantly higher relative abundance of bifidobacteria and *Faecalibacterium* spp. in IF infants at M12 compared with CF infants (Figure 2C). The occurrence of members of the genus *Escherichia*, which are clinically relevant bacteria, was comparable between breastfed and formula-fed infants at the age of 4 and 12 mo; a statistically significantly lower prevalence was noted in the HM group at month 24. The data presented here were confirmed by using a generalized linear model with a random intercept for individual subjects and fixed effects for visit time points, intervention groups, and study center, and additionally including an interaction term between visit time points and intervention groups (Supplemental Table 3c–d).

Intervention-associated taxonomic differences at M4 were linked to the presence of the probiotic species in the vast majority of IF-fed infants (93%), as analyzed by amplicon sequencing (Figure 3A), which indicates high compliance. This finding and the increased relative abundances of *Bifidobacterium* spp. observed by sequencing were confirmed by quantitative data using targeted qPCR assays (Figure 3B). The latter analysis also revealed a higher prevalence of *Clostridioides difficile* in formula-fed infants, without a significant difference between IF and CF. Regarding fecal milieu parameters, noticeable differences were observed at M4 due to the intervention. Compared to the CF group, butyrate and pH were lower, whereas fecal water content was higher in IF-fed infants. Thus, levels in the IF group resembled the values observed in breastfed infants (Figure 3C).

The fecal microbiota of breastfed infants clustered into different natural states

Due to the individual nature of gut microbiomes, the microbiota profiles of breastfed infants were analyzed using de novo clustering (Figure 4). At each time point, 2 (at M4 and M12) to 3 (at M24) distinct clusters of fecal microbiota profiles were observed (Figure 4A and Supplemental Table 4). The within-time point distance between the natural microbiota clusters was highest at M4, i.e., profiles tended to converge over time while still being distinct. To determine the influence of the synbiotic intervention at M4, M12, and M24, we calculated phylogenetic distances of all individual samples in each feeding group to the closest cluster medoid, i.e., the reference HM sample for the given cluster (orange dots in Figure 4A). At M4, microbiota profiles of infants in the IF groups were significantly closer to the reference HM profiles than those of CF infants (Figure 4B). At M12 and M24, no significant differences between breastfed and formula-fed infants were observed. The HM-microbiota clusters were accompanied by major differences in taxonomic composition (Figure 4C): samples within cluster 1 (M4-HM1) were dominated by members of the genus *Bacteroides* (phylum Bacteroidota) at the expense of *Bifidobacterium* (phylum Actinobacteriota), *Collinsella* (phylum Actinobacteriota), and *Escherichia* (phylum Proteobacteria). Interestingly, breastfed infants at M4 were devoid of *Akkermansia* (phylum Verrucomicrobiota; formerly Verrucomicrobia) and *Faecalibacterium* spp., which were detected in at least half of the infants at 12 and 24 months of age (Figure 4C). These data indicate that the early phylogenetic makeup of the fecal microbiota was influenced by the synbiotic intervention when considering inter-individual variability in natural microbiota profiles of breastfed infants.

Synbiotic intervention influenced microbiota states associated with CS

Considering the IF-associated changes in infant fecal microbiota observed at M4 (Figures 2–4), we further investigated this specific time point when the effects of influencing factors on the microbiota, such as complementary feeding, are less important (Figure 5). Two distinctly separated clusters (C1 and C2) of the fecal microbiota

FIGURE 4. Effects of the intervention in relation to the fecal microbiota profiles observed in breastfed infants during the first 2 years of life. (A) MDS plot based on de novo clustering. Orange dots indicate the samples selected as medoids, which best represent the overall microbiota phylogenetic profile within the corresponding cluster. (B) Phylogenetic distances between the fecal microbiota profile of any single infant in each feeding group within a given time point and the closest medoid of the reference cluster profile from the breastfed infants. (C) Significant differences in diversity and taxonomic composition between the infant gut microbiota profiles within each time point. Percentages below the respective *x*-axis indicate prevalence, i.e., the fraction of samples positive for the given taxon. The total number of infants within each cluster at the different time points is indicated in bold letters at the bottom of the graphs. Any orange dot indicates the value for the given medoid. Statistics: Stars (above the black lines linking the 2 groups with a significant difference) indicate differences in relative abundance using the Wilcoxon rank sum test. Crosses (below the lines) indicate differences in prevalence, i.e., the percentage of samples positive for the given taxon, as indicated below the respective *x*-axis and tested using Fisher's exact test. *, adjusted *P* value <0.05; **, <0.01; ***, <0.001. CF, control formula; HM; human milk; IF, intervention formula; M, months of age; MDS, multidimensional scaling.

were observed at M4 based on phylogenetic distances, mostly explained by the presence of Bacteroidota (higher in C1; violet bars) (Figure 5A). The C2 cluster was associated with a statistically significant higher prevalence of both CS infants in general (χ^2 P value = 6.1E-05) and those receiving IF (χ^2 P value = 0.000393), whereas the C1 cluster was associated with a higher prevalence of CF infants (χ^2 P values = 8.27E-05) (Figure 5B). Of note, HM feeding was equally represented in the 2 clusters (χ^2 P value = 0.888638). In terms of bacterial composition, C2 was characterized by a lower occurrence of *Bacteroides* and a higher occurrence of Firmicutes, Proteobacteria, and *Bifidobacterium* (Figure 5C). Comparison of the fecal milieu parameters between infants born via vaginal delivery or CS within each cluster showed no significant differences for acetate, propionate, fecal pH, and fecal water content (Figure 5C and Supplemental Figure 3). Infants born by CS in microbiota cluster C2 were characterized by higher fecal concentrations of butyrate and a lower prevalence of both *Bacteroides* and *Collinsella* spp. compared with infants in cluster C1 (Figure 5C).

Following the approach described above that consists of calculating similarities to reference HM medoid profiles, we found that the M4-specific effects of IF (Figure 4B) was associated primarily with infants presenting microbiota profile C2 (Figure 6A; right panel and Supplemental Table 4), inferring that the structure of the infant microbiota is associated with the magnitude of changes due to the synbiotic intervention. The major changes in diversity and composition, as well as gut milieu parameters observed in the IF group, were primarily related to infants with a C2-type microbiota. These changes included lower richness, lower relative abundances of *Erysipelatoclostridium* and members of the *R. gnavus* group (Figure 6B), and lower concentrations of fecal butyrate and pH (Figure 6C), resembling the profiles in HM infants. In contrast, relative abundances of *Bifidobacterium* spp. and *Lactobacillaceae,* as well as fecal concentrations of acetate, were higher in the IF group than in the CF group (Figure 6B and C).

Discussion

Microbial colonization of the intestine after birth is a central event that influences infant health with life-long consequences. It is therefore important to study factors (e.g., infant diet) that determine colonization processes. This clinical study investigated the effects of a synbiotic intervention with *L. fermentum* CECT5716 and GOS on the gut microbiota during early life in 540 infants.

Our observations on gut microbiota diversity and composition during the first year of life are in line with data from the scientific literature [7, 41, 42], e.g., low overall diversity and high relative abundances of bifidobacteria and *Enterobacteriaceae*. In addition, *Akkermansia* spp. and *Faecalibacterium* spp. were absent in breastfed infants at the age of 4 months and occurred at low prevalence (<10%) in formula-fed infants. However, these and other strictly anaerobic bacteria were detected in most infants in all feeding groups at later time points (M12 and M24), which agrees with common knowledge on gut ecosystem maturation [41, 43].

While most studies on infant microbiota focus on high-throughput profiling by sequencing, we could associate the development of stool microbiota structure with gut milieu parameters. Across all feeding groups, increasing pH and butyrate concentrations and decreasing fecal water content, acetate, sIgA, and calprotectin levels were observed at an older age (months 12 and 24). In a recent review of 14 clinical studies published between 1926 and 2017, increasing fecal pH from 5.0 to 6.5 over the past century was reported, along with the proposal that low fecal pH is a marker for gut health, due to its association with increased occurrence of bifidobacteria [44]. Fecal pH in breastfed infants was previously reported to be lower than that in formula-fed infants [45, 46]. Here, we observed lower pH levels due to the synbiotic intervention compared with the control group.

The fecal concentrations of SCFAs measured here are in the range of data from the scientific literature [43, 47, 48]. In accordance with the dominance of bifidobacteria at an early age, levels of acetate were higher at the age of 4 months (whole cohort) and highest in feces of infants fed synbiotic IF (though the latter difference was not statistically significant). This might be important considering that previous studies reported an association between higher fecal levels of acetate at 3 months of age and lower risk of atopic respiratory diseases [49, 50]. Low concentrations of butyrate at M4, as detected in our study, fit with the usual concept of microbiota maturation with strictly anaerobic species dominating the ecosystem later during the first year of life [41, 43]. In this respect, infants in the synbiotic group resembled breastfed infants, i.e., with lower concentrations of butyrate. At M12, the intervention led to a higher relative abundance of faecalibacteria, which are important butyrate producers in the human gut, at levels similar to breastfed infants. Previous data from the PASTURE study (Protection against Allergy; Study in Rural Environments) proposed an inverse association between fecal butyrate and childhood asthma at 12 months of age [4].

Effects of the synbiotic intervention across the entire study population included increased relative abundances of bifidobacteria at M4 and M12, which confirms results from a previous study using an infant formula supplemented with *L. fermentum* CECT5716 and GOS [22]. In the present study, the synbiotic intervention was linked to decreased relative abundances of *Blautia* spp. and *R. gnavus*-related bacteria in infants at M4, resembling levels observed in breastfed controls. Although *R. gnavus* is a common species within the human intestine [42, 51], changes in its occurrence have been linked to the development of allergic diseases, either negatively [52, 53] or positively [54]. Lower enteric colonization by *Blautia* spp. due to synbiotic intervention at M4 reflected the situation in breastfed infants as this genus is usually associated with a more mature microbiota composition later in life [42, 55]. More work is required to understand the functional role and health consequences of different colonization dynamics of strict anaerobes such as *Blautia*, *Ruminoccocus*, and *Faecalibacterium* spp. in early life.

Recently, independent studies proposed the existence of natural microbiota states in infants at a young age [55–58]. Galazzo et al. [55] identified 6 microbiota clusters using stool samples from 312 breastfed infants between the ages of 5 to 31 weeks, albeit 1 to 2 clusters attracted most of the infants at each time point. A very recent, large-scale study by Xiao et al. [58] described the presence of 4 enterotype-like clusters based on samples from 2000 infants; these clusters were associated with different maturation states of the microbiota. In the present study, we highlight the existence of natural gut microbiota states by identifying 2 natural community profiles in breastfed infants at M4 and M12 and 3 clusters at M24. Interestingly, the data suggest that the effects of the synbiotic intervention early in life might be associated with these natural states. Microbiota changes due to the intervention (e.g., decreased richness, lower relative abundances of erysipelatoclostridia, *R. gnavus* group, butyrate concentrations, and increased relative abundance of *Bifidobacterium* spp.) were more pronounced in the cluster characterized by lower occurrence of *Bacteroides* spp. at M4. In contrast to previous reports [59], enhanced effects of IF was not due to better engraftment of the probiotic in the corresponding infants as

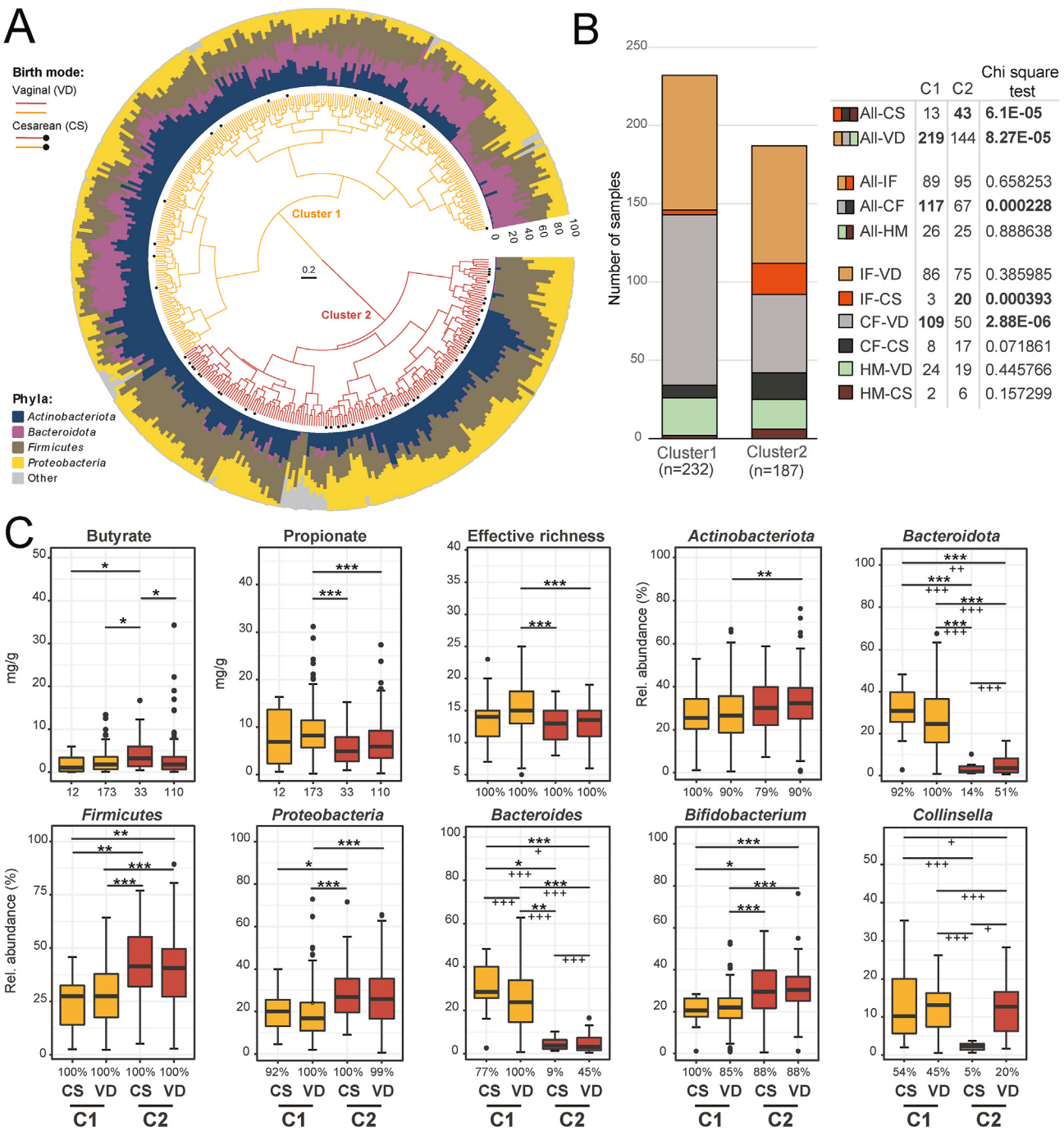


FIGURE 5. Fecal microbiota clusters at month 4 of age linked to birth mode. (A) Cladogram of relative sample similarity based on generalized UniFrac distances for all samples at M4. The 2 microbiota clusters appear in orange (C1) and red (C2). Black dots indicate infants born by Cesarean section (CS). The colored stacked bars surrounding each branch indicate the corresponding microbiota composition at the phylum level (0%–100% relative abundance). (B) Prevalence of infants within each microbiota cluster with respect to the mode of birth and feeding group; significance from deviation from the null hypothesis of equal representation of categories across the 2 clusters is given in the right ($\chi 2$ test). (C) Significant fecal milieu and microbiota parameters across the 2 microbiota clusters (C1, C2) and mode of birth (CS, VD). For butyrate and propionate, the numbers in parentheses below the *x*-axis correspond to the number of values available. In all other graphs, percentages below the respective *x*-axis indicate prevalence, i.e., the fraction of samples positive for the given taxon relative to the respective total number of infants: C1/CS, $n = 13$; C1/VD $n = 219$; C2/CS, $n = 43$; C2/VD, $n = 144$. Statistics: Stars (above the black lines linking the 2 groups with a significant difference) indicate differences in relative abundance using the Wilcoxon rank sum test. Crosses (below the lines) indicate differences in prevalence, i.e., the percentage of samples positive for the given taxon, as indicated below the respective *x*-axis and tested using Fisher's exact test. *, adjusted *P* value <0.05; **, <0.01; ***, <0.001. The total numbers of infants per feeding group are indicated in bold letters at the bottom of the graphs. The HM group is a non-randomized reference group. CF, control formula; HM; human milk; IF, intervention formula.

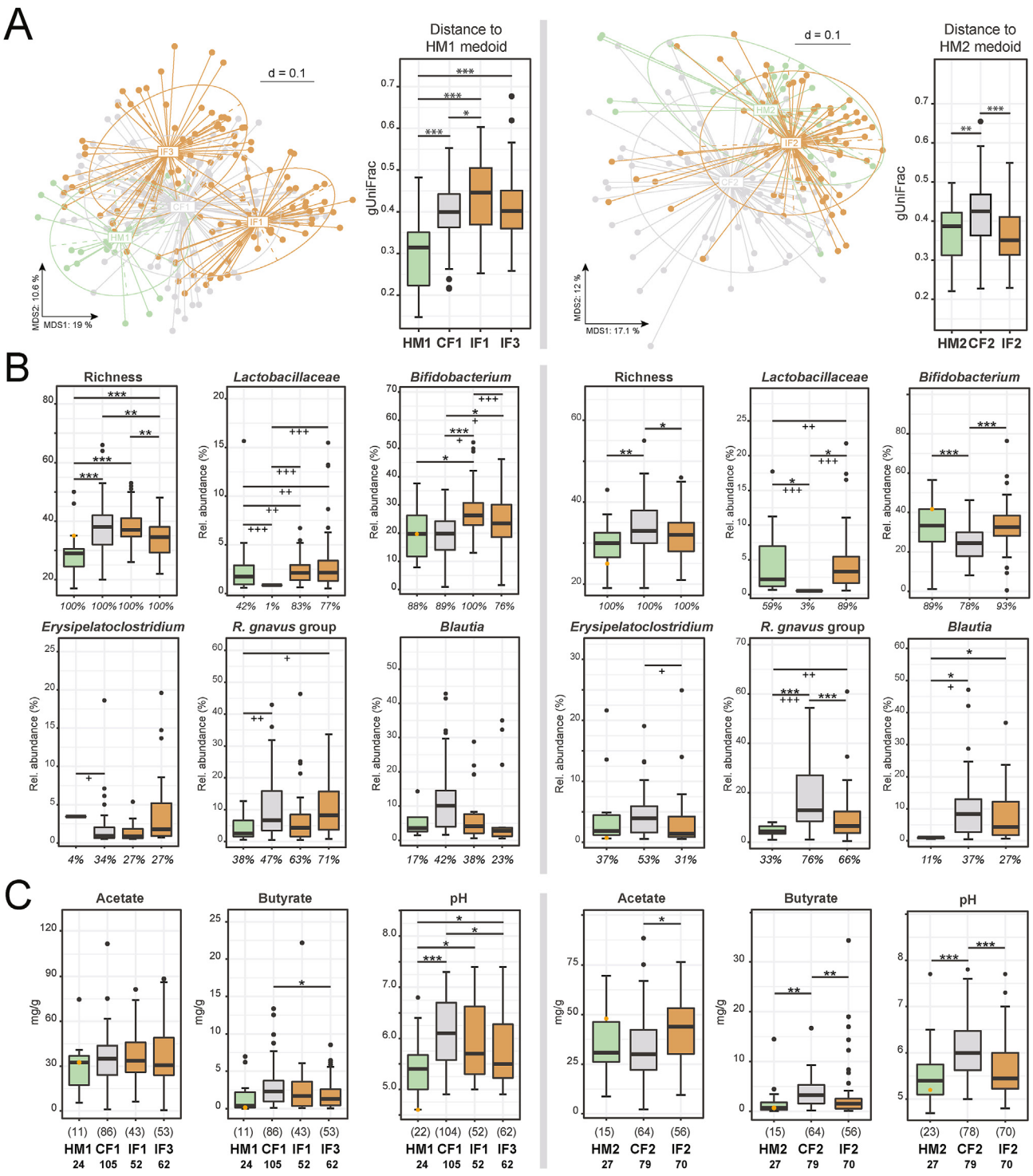


FIGURE 6. Effects of the synbiotic intervention depending on microbiota profiles. (A) MDS plots of microbiota profiles based on generalized UniFrac (gUniFrac) distances. All profiles within each feeding group were clustered de novo. The clusters were then plotted separately depending on their proximity to one of the 2 reference clusters from breastfed infants (HM1 or HM2; similar to the numbering cluster 1 and 2 in Figure 5). (B) Corresponding changes in diversity and composition. Percentages below the respective *x*-axis indicate prevalence, i.e., the fraction of samples positive for the given taxon relative to the respective total number of infants. The total number of infants within each cluster at the different time points is indicated in bold letters at the bottom of the graphs. (C) Changes in fecal milieu parameters. The numbers in parentheses below the *x*-axis correspond to the number of values available (i.e., data were not available for the remaining samples). Statistics: Stars (above the black lines linking the 2 groups with a significant difference) indicate differences in relative abundance using the Wilcoxon rank sum test. Crosses (below the lines) indicate differences in prevalence, i.e., the percentage of samples positive for the given taxon, as indicated below the respective *x*-axis and tested using Fisher's exact test. *, adjusted *P* value <0.05; **, <0.01; ***, <0.001. CF, control formula; HM; human milk; IF, intervention formula; MDS, multidimensional.

nearly all infants in the CF group were deprived of lactobacilli, and the infants in the IF group were characterized by higher prevalence and relative abundances of the target species. This effect was independent of their natural microbiota state. Notably, the prevalence of infants born by CS was higher in this microbiota cluster characterized by enhanced responses to treatment. Although this does not represent a direct link between the efficacy of the synbiotic intervention and the mode of birth, it draws attention to the necessity of better understanding the forces that shape microbiota structures at an early age and the factors that influence them (e.g., mode of birth), to design the best possible alternative feeding strategies when breastfeeding is not possible.

Few studies have previously reported positive effects of nutritional interventions with probiotics or synbiotics in CS-born infants. One recent trial highlighted the ability of a 3-month intervention with a multi-species probiotic (containing 2 *Lacticaseibacillus rhamnosus* strains) to alleviate CS- and antibiotics-induced alterations of the gut microbiota [60]. Another study reported temporary efficacy (during intervention only) of a synbiotic consisting of GOS/FOS (fructooligosaccharides) and *Bifidobacterium breve* M-16V in lowering fecal pH and increasing relative abundances of bifidobacteria in CS-born infants [61]. However, as effects are always dependent on the synbiotic formulation (e.g., the bacterial strain used), it is difficult to generalize results. Therefore, state-of-the-art clinical studies at larger scales will be needed to clinically demonstrate the effects of specific synbiotic products in regulating the infant gut microbiota and health, especially in the context of birth after CS.

Of note, this randomized clinical study has 3 major limitations: First, due to its design and the gut microbiota being a secondary outcome, stool samples from the infants at baseline visit prior to formula intervention were not collected. Therefore, the results describe associations between the microbiota states of the infants and the effects of the intervention; a causal relationship could not be determined. Second, following up with the infants during the first 2 years of life allowed us to observe the development of gut microbiota and associated factors over a relatively long period of time. However, the number of available samples decreased over time, as indicated in each figure and corresponding legend. Hence, attrition bias due to the loss of participants over the 2-y time period cannot be fully excluded. Third, this manuscript focused primarily on time point-specific differences in the microbiota due to the known major ecological changes occurring within the ecosystem in the first 2 years of life. The main outcomes were confirmed by using generalized linear models to assess changes in the microbiota and other data in a longitudinal manner. Data processing and statistical approaches to comprehensively analyze the factors influencing infant gut microbiota trajectories will provide additional insights into future studies.

In conclusion, in the present study, synbiotic intervention influenced the gut microbiota and milieu parameters during early life to resemble some major characteristics found in breastfed infants (higher relative abundances of bifidobacteria, lower richness, lower fecal pH and butyrate concentrations), and effects depended on the ecosystem profile of the infants. Further analysis of clinical parameters associated with such changes is now needed to test the potential for disease prevention (e.g., diarrhea or respiratory tract infections). Furthermore, specific randomized, controlled studies that focus on infants born by CS and how early nutrition can support the beneficial development of their microbiota are required.

Acknowledgements

We are very grateful to our colleagues at Biofortis for their great support with study coordination, site care, and lab work and methods. We are also thankful to all study sites for excellent participant care, especially to the participating families, for their willingness to join the study and their compliance. We want to thank Dr. Julia Günther and Dr. Kirsten Berding-Harold (HiPP GmbH) for their excellent support in editing the manuscript.

The authors' responsibilities were as follows; HP and CH: designed research (project conception, development of overall research plan, and study oversight). IL and EI: analyzed data, performed bioinformatics analyses, and generated data and readouts. JPKR: performed statistical analyses. IL and TC: interpreted the data. MS and TC: coordinated the project and data analysis. IL, MS, and TC: wrote the paper. CH: reviewed and revised the manuscript. All authors have read and approved the final manuscript as submitted and agreed to be accountable for all aspects of the work.

This study design was planned and written by HiPP GmbH & Co. Vertrieb KG. Infant formulae were provided by HiPP GmbH & Co. Vertrieb KG. Recruiting and sample collection were done by Biofortis by order of HiPP GmbH & Co. Vertrieb KG. Sample analysis and interpretation were free of sponsor involvement or restrictions regarding publication.

Data Availability

The raw amplicon data generated in the context of the present study have been submitted to the European Nucleotide Archive and are available under the project accession number PRJEB47935.

Funding

TC and IL received funding from HiPP GmbH & Co. Vertrieb KG, Pfaffenhofen, Germany, to perform data analysis. TC received funding from the DFG (German Research Foundation), Project 403224013 "SFB1382" and Project 460129525 "NFDI4Microbiota." CH and MS are employees at HiPP GmbH & Co. Vertrieb KG, Pfaffenhofen, Germany, Research and Development. HP received consultation fees from HiPP and supervised the study. JPKR is an employee of Staburo GmbH, Munich, Germany, subcontracted by HiPP GmbH for statistical services. EI reports no conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ajcnut.2022.11.012.

References

[1] M.C. de Goffau, S. Lager, U. Sovio, F. Gaccioli, E. Cook, S.J. Peacock, et al., Human placenta has no microbiome but can contain potential pathogens, Nature 572 (7769) (2019) 329–334, https://doi.org/10.1038/s41586-019-1451-5.

[2] J. Walter, M.W. Hornef, A philosophical perspective on the prenatal in utero microbiome debate, Microbiome 9 (1) (2021) 5–6, https://doi.org/10.1186/s40168-020-00979-7.

[3] H. Renz, B.D. Adkins, S. Bartfeld, R.S. Blumberg, D.L. Farber, J. Garssen, et al., The neonatal window of opportunity-early priming for life, J Allergy Clin Immunol 141 (4) (2018) 1212–1214, https://doi.org/10.1016/j.jaci.2017.11.019.

[4] M. Depner, D.H. Taft, P.V. Kirjavainen, K.M. Kalanetra, A.M. Karvonen, S. Peschel, et al., Maturation of the gut microbiome during the first year of life contributes to the protective farm effect on childhood asthma, Nat Med 26 (11) (2020) 1766–1775, https://doi.org/10.1038/s41591-020-1095-x.

[5] M.J. Ege, I. Herzum, G. Buchele, S. Krauss-Etschmann, R.P. Lauener, M. Roponen, et al., Prenatal exposure to a farm environment modifies atopic

sensitization at birth, J Allergy Clin Immunol 122 (2) (2008) 407–412, https://doi.org/10.1016/j.jaci.2008.06.011, e1-412.
[6] P.V. Kirjavainen, A.M. Karvonen, R.I. Adams, M. Taubel, M. Roponen, P. Tuoresmaki, et al., Farm-like indoor microbiota in non-farm homes protects children from asthma development, Nat Med 25 (7) (2019) 1089–1095, https://doi.org/10.1038/s41591-019-0469-4.
[7] J. Roswall, L.M. Olsson, P. Kovatcheva-Datchary, S. Nilsson, V. Tremaroli, M.C. Simon, et al., Developmental trajectory of the healthy human gut microbiota during the first 5 years of life, Cell Host Microbe 29 (5) (2021) 765–776, https://doi.org/10.1016/j.chom.2021.02.021, e3.
[8] M.R. Charbonneau, D. O'Donnell, L.V. Blanton, S.M. Totten, J.C. Davis, M.J. Barratt, et al., Sialylated milk oligosaccharides promote microbiota-dependent growth in models of infant undernutrition, Cell 164 (5) (2016) 859–871, https://doi.org/10.1016/j.cell.2016.01.024.
[9] A. Marcobal, M. Barboza, E.D. Sonnenburg, N. Pudlo, E.C. Martens, P. Desai, et al., Bacteroides in the infant gut consume milk oligosaccharides via mucus-utilization pathways, Cell Host Microbe 10 (5) (2011) 507–514, https://doi.org/10.1016/j.chom.2011.10.007.
[10] D.A. Sela, D.A. Mills, Nursing our microbiota: molecular linkages between bifidobacteria and milk oligosaccharides, Trends Microbiol 18 (7) (2010) 298–307, https://doi.org/10.1016/j.tim.2010.03.008.
[11] S. Moossavi, S. Sepehri, B. Robertson, L. Bode, S. Goruk, C.J. Field, et al., Composition and variation of the human milk microbiota are influenced by maternal and early-life factors, Cell Host Microbe 25 (2) (2019) 324–335, https://doi.org/10.1016/j.chom.2019.01.011, e4.
[12] K. Murphy, D. Curley, T.F. O'Callaghan, C.A. O'Shea, E.M. Dempsey, P.W. O'Toole, et al., The composition of human milk and infant faecal microbiota over the first three months of life: a pilot study, Sci Rep 7 (2017) 40597–40598, https://doi.org/10.1038/srep40597.
[13] K.E. Lyons, C.A. Ryan, E.M. Dempsey, R.P. Ross, C. Stanton, Breast milk, a source of beneficial microbes and associated benefits for infant health, Nutrients 12 (4) (2020) 1039, https://doi.org/10.3390/nu12041039.
[14] M.G. Dominguez-Bello, K.M. De Jesus-Laboy, N. Shen, L.M. Cox, A. Amir, A. Gonzalez, et al., Partial restoration of the microbiota of cesarean-born infants via vaginal microbial transfer, Nat Med 22 (3) (2016) 250–253, https://doi.org/10.1038/nm.4039.
[15] K. Korpela, O. Helve, K.L. Kolho, T. Saisto, K. Skogberg, E. Dikareva, et al., Maternal fecal microbiota transplantation in Cesarean-born infants rapidly restores normal gut microbial development: a proof-of-concept study, Cell 183 (2) (2020) 324–334, https://doi.org/10.1016/j.cell.2020.08.047, e5.
[16] J. Zheng, S. Wittouck, E. Salvetti, C. Franz, H.M.B. Harris, P. Mattarelli, et al., A taxonomic note on the genus *Lactobacillus*: description of 23 novel genera, emended description of the genus *Lactobacillus* Beijerinck 1901, and union of Lactobacillaceae and Leuconostocaceae, Int J Syst Evol Microbiol 70 (4) (2020) 2782–2858, https://doi.org/10.1099/ijsem.0.004107.
[17] I. Martínez, J.C. Stegen, M.X. Maldonado-Gómez, A.M. Eren, P.M. Siba, A.R. Greenhill, et al., The gut microbiota of rural Papua New Guineans: composition, diversity patterns, and ecological processes, Cell Rep 11 (4) (2015) 527–538, https://doi.org/10.1016/j.celrep.2015.03.049.
[18] E.D. Sonnenburg, S.A. Smits, M. Tikhonov, S.K. Higginbottom, N.S. Wingreen, J.L. Sonnenburg, Diet-induced extinctions in the gut microbiota compound over generations, Nature 529 (7585) (2016) 212–215, https://doi.org/10.1038/nature16504.
[19] R. Martín, S. Langa, C. Reviriego, E. Jimínez, M.L. Marín, J. Xaus, et al., Human milk is a source of lactic acid bacteria for the infant gut, J Pediatr 143 (6) (2003) 754–758, https://doi.org/10.1016/j.jpeds.2003.09.028.
[20] R. Martín, M. Olivares, M.L. Marín, L. Fernández, J. Xaus, J.M. Rodríguez, Probiotic potential of 3 Lactobacilli strains isolated from breast milk, J Hum Lact 21 (1) (2005) 8–17, https://doi.org/10.1177/0890334404272393.
[21] M. Gil-Campos, M. López, M.V. Rodriguez-Benítez, J. Romero, I. Roncero, M.D. Linares, et al., *Lactobacillus fermentum* CECT 5716 is safe and well tolerated in infants of 1-6 months of age: a randomized controlled trial, Pharmacol Res 65 (2) (2012) 231–238, https://doi.org/10.1016/j.phrs.2011.11.016.
[22] J. Maldonado, F. Cañabate, L. Sempere, F. Vela, A.R. Sánchez, E. Narbona, et al., Human milk probiotic *Lactobacillus fermentum* CECT5716 reduces the incidence of gastrointestinal and upper respiratory tract infections in infants, J Pediatr Gastroenterol Nutr 54 (1) (2012) 55–61, https://doi.org/10.1097/MPG.0b013e3182333f18.
[23] J. Maldonado, M. Gil-Campos, J.A. Maldonado-Lobón, M.R. Benavides, K. Flores-Rojas, R. Jaldo, et al., Evaluation of the safety, tolerance and efficacy of 1-year consumption of infant formula supplemented with *Lactobacillus fermentum* CECT5716 Lc40 or *Bifidobacterium breve* CECT7263: a randomized controlled trial, BMC Pediatr 19 (1) (2019) 361–362, https://doi.org/10.1186/s12887-019-1753-7.
[24] J.A. Maldonado-Lobón, M. Gil-Campos, J. Maldonado, E. López-Huertas, K. Flores-Rojas, A.D. Valero, et al., Long-term safety of early consumption of *Lactobacillus fermentum* CECT5716: a 3-year follow-up of a randomized controlled trial, Pharmacol Res 95-96 (2015) 12–19, https://doi.org/10.1016/j.phrs.2015.01.006.
[25] X.M. Ben, X.Y. Zhou, W.H. Zhao, W.L. Yu, W. Pan, W.L. Zhang, et al., Supplementation of milk formula with galacto-oligosaccharides improves intestinal micro-flora and fermentation in term infants, Chin Med J (Engl). 117 (6) (2004) 927–931.
[26] S. Fanaro, B. Marten, R. Bagna, V. Vigi, C. Fabris, L. Peña-Quintana, et al., Galacto-oligosaccharides are bifidogenic and safe at weaning: a double-blind randomized multicenter study, J Pediatr Gastroenterol Nutr 48 (1) (2009) 82–88, https://doi.org/10.1097/MPG.0b013e31817b6dd2.
[27] C. Sierra, M.J. Bernal, J. Blasco, R. Martínez, J. Dalmau, I. Ortuño, et al., Prebiotic effect during the first year of life in healthy infants fed formula containing GOS as the only prebiotic: a multicentre, randomised, double-blind and placebo-controlled trial, Eur J Nutr 54 (1) (2015) 89–99, https://doi.org/10.1007/s00394-014-0689-9.
[28] T. Williams, Y. Choe, P. Price, G. Katz, F. Suarez, C. Paule, et al., Tolerance of formulas containing prebiotics in healthy, term infants, J Pediatr Gastroenterol Nutr 59 (5) (2014) 653–658, https://doi.org/10.1097/MPG.0000000000000513.
[29] A. Klindworth, E. Pruesse, T. Schweer, J. Peplies, C. Quast, M. Horn, et al., Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies, Nucleic Acids Res 41 (1) (2013) e1–2, https://doi.org/10.1093/nar/gks808.
[30] R.C. Edgar, UNOISE2: improved error-correction for Illumina 16S and ITS amplicon sequencing, bioRxiv (2016), 081257, https://doi.org/10.1101/081257.
[31] R.C. Edgar, Search and clustering orders of magnitude faster than BLAST, Bioinformatics 26 (19) (2010) 2460–2461, https://doi.org/10.1093/bioinformatics/btq461.
[32] I. Lagkouvardos, D. Joseph, M. Kapfhammer, S. Giritli, M. Horn, D. Haller, et al., IMNGS: a comprehensive open resource of processed 16S rRNA microbial profiles for ecology and diversity studies, Sci Rep 6 (1) (2016) 33721–33722, https://doi.org/10.1038/srep33721.
[33] E. Pruesse, J. Peplies, F.O. Glöckner, SINA: accurate high-throughput multiple sequence alignment of ribosomal RNA genes, Bioinformatics 28 (14) (2012) 1823–1829, https://doi.org/10.1093/bioinformatics/bts252.
[34] C. Quast, E. Pruesse, P. Yilmaz, J. Gerken, T. Schweer, P. Yarza, et al., The SILVA ribosomal RNA gene database project: improved data processing and web-based tools, Nucleic Acids Res 41 (2013) D590–D596, https://doi.org/10.1093/nar/gks1219.
[35] S.H. Yoon, S.M. Ha, S. Kwon, J. Lim, Y. Kim, H. Seo, et al., Introducing EzBioCloud: a taxonomically united database of 16S rRNA gene sequences and whole-genome assemblies, Int J Syst Evol Microbiol 67 (5) (2017) 1613–1617, https://doi.org/10.1099/ijsem.0.001755.
[36] S. Kumar, G. Stecher, M. Li, C. Knyaz, K. Tamura, MEGA X: molecular evolutionary genetics analysis across computing platforms, Mol Biol Evol 35 (6) (2018) 1547–1549, https://doi.org/10.1093/molbev/msy096.
[37] I. Lagkouvardos, S. Fischer, N. Kumar, T. Clavel, Rhea: a transparent and modular R pipeline for microbial profiling based on 16S rRNA gene amplicons, PeerJ 5 (2017) e2836–e2837, https://doi.org/10.7717/peerj.2836.
[38] S. Reitmeier, T.C.A. Hitch, N. Treichel, N. Fikas, B. Hausmann, A.E. Ramer-Tait, et al., Handling of spurious sequences affects the outcome of high-throughput 16S rRNA gene amplicon profiling, ISME Commun 1 (1) (2021) 31–32, https://doi.org/10.1038/s43705-021-00033-z.
[39] J. Chen, K. Bittinger, E.S. Charlson, C. Hoffmann, J. Lewis, G.D. Wu, et al., Associating microbiome composition with environmental covariates using generalized UniFrac distances, Bioinformatics 28 (16) (2012) 2106–2113, https://doi.org/10.1093/bioinformatics/bts342.
[40] E. Intze, I. Lagkouvardos, DivCom: a tool for systematic partition of groups of microbial profiles into intrinsic subclusters and distance-based subgroup comparisons, Front Bioinformatics 2 (2022) 864382–864383, https://doi.org/10.3389/fbinf.2022.864382.
[41] M. Bazanella, T.V. Maier, T. Clavel, I. Lagkouvardos, M. Lucio, M.X. Maldonado-Gòmez, et al., Randomized controlled trial on the impact of early-life intervention with bifidobacteria on the healthy infant fecal microbiota and metabolome, Am J Clin Nutr 106 (5) (2017) 1274–1286, https://doi.org/10.3945/ajcn.117.157529.
[42] D. Podlesny, W.F. Fricke, Strain inheritance and neonatal gut microbiota development: a meta-analysis, Int J Med Microbiol 311 (3) (2021) 151483–151484, https://doi.org/10.1016/j.ijmm.2021.151483.
[43] N. Tsukuda, K. Yahagi, T. Hara, Y. Watanabe, H. Matsumoto, H. Mori, et al., Key bacterial taxa and metabolic pathways affecting gut short-chain fatty acid profiles in early life, ISME J 15 (9) (2021) 2574–2590, https://doi.org/10.1038/s41396-021-00937-7.

[44] B.M. Henrick, A.A. Hutton, M.C. Palumbo, G. Casaburi, R.D. Mitchell, M.A. Underwood, et al., Elevated fecal pH indicates a profound change in the breastfed infant gut microbiome due to reduction of Bifidobacterium over the past century, mSphere 3 (2) (2018), https://doi.org/10.1128/mSphere.00041-18 e00041–18.
[45] K. Ogawa, R.A. Ben, S. Pons, M.I. de Paolo, L. Bustos Fernández, Volatile fatty acids, lactic acid, and pH in the stools of breast-fed and bottle-fed infants, J Pediatr Gastroenterol Nutr 15 (3) (1992) 248–252, https://doi.org/10.1097/00005176-199210000-00004.
[46] C.R. Kok, B. Brabec, M. Chichlowski, C.L. Harris, N. Moore, J.L. Wampler, et al., Stool microbiome, pH and short/branched chain fatty acids in infants receiving extensively hydrolyzed formula, amino acid formula, or human milk through two months of age, BMC Microbiol 20 (1) (2020) 337–338, https://doi.org/10.1186/s12866-020-01991-5.
[47] O.C. Thompson-Chagoyan, M. Fallani, J. Maldonado, J.M. Vieites, S. Khanna, C. Edwards, et al., Faecal microbiota and short-chain fatty acid levels in faeces from infants with cow's milk protein allergy, Int Arch Allergy Immunol 156 (3) (2011) 325–332, https://doi.org/10.1159/000323893.
[48] R. Berni Canani, N. Sangwan, A.T. Stefka, R. Nocerino, L. Paparo, R. Aitoro, et al., *Lactobacillus rhamnosus* GG-supplemented formula expands butyrate-producing bacterial strains in food allergic infants, ISME J 10 (3) (2016) 742–750, https://doi.org/10.1038/ismej.2015.151.
[49] M.C. Arrieta, L.T. Stiemsma, P.A. Dimitriu, L. Thorson, S. Russell, S. Yurist-Doutsch, et al., Early infancy microbial and metabolic alterations affect risk of childhood asthma, Sci Transl Med 7 (307) (2015), https://doi.org/10.1126/scitranslmed.aab2271, 307ra152.
[50] M.C. Arrieta, A. Arévalo, L. Stiemsma, P. Dimitriu, M.E. Chico, S. Loor, et al., Associations between infant fungal and bacterial dysbiosis and childhood atopic wheeze in a nonindustrialized setting, J Allergy Clin Immunol 142 (2) (2018) 424–434, https://doi.org/10.1016/j.jaci.2017.08.041, e10.
[51] V. Sagheddu, V. Patrone, F. Miragoli, E. Puglisi, L. Morelli, Infant early gut colonization by Lachnospiraceae: high frequency *of Ruminococcus gnavus*, Front Pediatr 4 (2016) 57–58, https://doi.org/10.3389/fped.2016.00057.
[52] H. Zheng, H. Liang, Y. Wang, M. Miao, T. Shi, F. Yang, et al., Altered gut microbiota composition associated with eczema in infants, PLOS ONE 11 (11) (2016), e0166026–01, https://doi.org/10.1371/journal.pone.0166026.
[53] H.H. Chua, H.C. Chou, Y.L. Tung, B.L. Chiang, C.C. Liao, H.H. Liu, et al., Intestinal dysbiosis featuring abundance of *Ruminococcus gnavus* associates with allergic diseases in infants, Gastroenterology 154 (1) (2018) 154–167, https://doi.org/10.1053/j.gastro.2017.09.006.
[54] M.J. Lee, M.J. Kang, S.Y. Lee, E. Lee, K. Kim, S. Won, et al., Perturbations of gut microbiome genes in infants with atopic dermatitis according to feeding type, J Allergy Clin Immunol 141 (4) (2018) 1310–1319, https://doi.org/10.1016/j.jaci.2017.11.045.
[55] G. Galazzo, N. van Best, L. Bervoets, I.O. Dapaah, P.H. Savelkoul, M.W. Hornef, et al., Development of the microbiota and associations with birth mode, diet, and atopic disorders in a longitudinal analysis of stool samples, collected from infancy through early childhood, Gastroenterology 158 (6) (2020) 1584–1596, https://doi.org/10.1053/j.gastro.2020.01.024.
[56] R.M. Pace, J.E. Williams, B. Robertson, K.A. Lackey, C.L. Meehan, W.J. Price, et al., Variation in human milk composition is related to differences in milk and infant fecal microbial communities, Microorganisms 9 (6) (2021) 1153, https://doi.org/10.3390/microorganisms9061153.
[57] C.J. Stewart, N.J. Ajami, J.L. O'Brien, D.S. Hutchinson, D.P. Smith, M.C. Wong, et al., Temporal development of the gut microbiome in early childhood from the TEDDY study, Nature 562 (7728) (2018) 583–588, https://doi.org/10.1038/s41586-018-0617-x.
[58] L. Xiao, J. Wang, J. Zheng, X. Li, F. Zhao, Deterministic transition of enterotypes shapes the infant gut microbiome at an early age, Genome Biol 22 (1) (2021) 243–244, https://doi.org/10.1186/s13059-021-02463-3.
[59] M.X. Maldonado-Gómez, I. Martínez, F. Bottacini, A. O'Callaghan, M. Ventura, D. van Sinderen, et al., Stable engraftment of *Bifidobacterium longum* AH1206 in the human gut depends on individualized features of the resident microbiome, Cell Host Microbe 20 (4) (2016) 515–526, https://doi.org/10.1016/j.chom.2016.09.001.
[60] K. Korpela, A. Salonen, O. Vepsäläinen, M. Suomalainen, C. Kolmeder, M. Varjosalo, et al., Probiotic supplementation restores normal microbiota composition and function in antibiotic-treated and in caesarean-born infants, Microbiome 6 (1) (2018) 182–183, https://doi.org/10.1186/s40168-018-0567-4.
[61] N. Phavichitr, S. Wang, S. Chomto, R. Tantibhaedhyangkul, A. Kakourou, S. Intarakhao, et al., Impact of synbiotics on gut microbiota during early life: a randomized, double-blind study, Sci Rep 11 (1) (2021) 3534–3535, https://doi.org/10.1038/s41598-021-83009-2.