Intestinal microbiota in infants at high risk for allergy: Effects of prebiotics and role in eczema development

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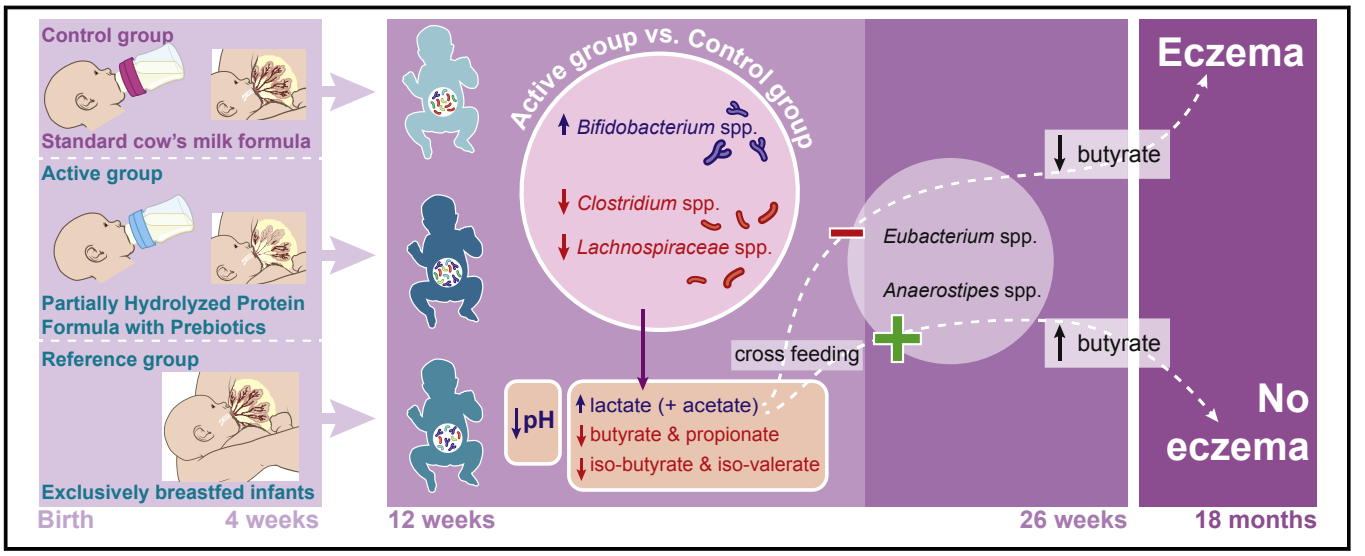
GRAPHICAL ABSTRACT



Background: Development of the gut microbiota in infancy is important in maturation of the immune system. Deviations in colonization patterns have been associated with allergic manifestations such as eczema, but exact microbiome dysfunctions underlying allergies remain unclear. We studied the gut microbiota of 138 infants at increased risk of allergy, participating in a clinical trial investigating the effectiveness of a partially hydrolyzed protein formula supplemented with nondigestible oligosaccharides on the prevention of eczema.

Objective: The effects of interventions and breast-feeding on fecal microbiota were investigated. Additionally, we aimed to identify microbial patterns associated with the onset of eczema.

Methods: Bacterial taxonomic compositions in the first 26 weeks of life were analyzed by using 16S rRNA gene sequencing. Additionally, fecal pH and microbial metabolite levels were measured.

Results: Fecal microbial composition, metabolites, and pH of infants receiving partially hydrolyzed protein formula supplemented with nondigestible oligosaccharides was closer to that of breast-fed infants than that of infants receiving standard cow's milk formula. Infants with eczema by 18 months showed discordant development of bacterial genera of Enterobacteriaceae and *Parabacteroides* species in the first 26 weeks, as well as decreased acquisition of lactate-utilizing bacteria producing butyrate, namely *Eubacterium* and *Anaerostipes* species, supported by increased lactate and decreased butyrate levels.

Conclusions: We showed that a partially hydrolyzed protein infant formula with specific prebiotics modulated the gut microbiota closer to that of breast-fed infants. Additionally, we identified a potential link between microbial activity and onset of eczema, which might reflect a suboptimal implementation of gut microbiota at specific developmental stages in infants at high risk for allergy. (J Allergy Clin Immunol 2018;141:1334-42.)

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Gut microbial development in the first years of life occurs concomitantly to the development of our cognitive, metabolic, and immune systems and forms an interactive signaling network.[1] The gradual diversification toward a relatively stable adult-like composition is a dynamic process influenced by several environmental factors, such as birth mode, gestational age at birth, and early-life nutrition. Alterations in the early colonization process, such as through antibiotic use or cesarean section delivery, have been associated with development of allergic manifestations later in life, but the exact microbiome dysfunction underlying this disease remains unclear.[2]

Eczema is typically the first allergic manifestation to appear, and its incidence is still increasing in many countries around the world. Considering the concurrent maturation of the immune system, the succession of species into a stable adult-type community, a process naturally guided by human milk, might be critically important. Breast-feeding is thought to protect against the development of allergy through the presence of allergens and immune mediators in human milk that are absent from artificial milk,[3] as well as through the presence of human milk oligosaccharides stimulating a gut microbiota that might favor tolerance induction.[4]

Therefore several studies have been performed with different types of infant formulas enriched with nondigestible oligosaccharides, which are known as prebiotics. Prebiotics typically reach the colon intact and selectively stimulate the growth and activity of specific beneficial members of the microbiota.[5,6] Interestingly, significant reductions in eczema risk were observed in intervention trials with infant formulas supplemented with specific prebiotics.[7-9]

The present study arises from a parent registered study (PATCH trial) investigating the effects of a partially hydrolyzed formula containing specific oligosaccharides (pHF-OS) on the prevention of eczema in infants at increased risk of allergy. pHF-OS was shown to induce hypoantigenic and immunomodulatory effects, including increased regulatory T-cell numbers, but did not reduce eczema incidence by 12 or 18 months when compared with that in infants receiving standard cow's milk formula.[10]

In this work 16S rRNA gene sequencing was applied to obtain an in-depth characterization of the microbiota composition of feces collected at 4 and 26 weeks of age in a subset of vaginally born infants, including breast-fed infants (n = 30) and infants randomized to receive pHF-OS (n = 51) or standard cow's milk formula (control subjects, n = 57). In addition, fecal pH and levels of lactate and short-chain fatty acids (SCFAs) were determined in stool specimens collected at 4, 12, and 26 weeks of age.

There were 2 primary aims of this study: (1) to investigate whether a pHF-OS could modulate the developing gut microbiota closer to that of breast-fed infants and (2) to identify patterns in the developing gut microbiota that might be implicated in the onset of eczema. The findings in this study confirm the major influence of early-life nutrition on assembly of the gut microbiota and provide new insights into how deviations in this assembly are associated with eczema development.

Abbreviations used

LUB:	Lactate-utilizing and butyrate-producing bacterium
MCPT:	Monte Carlo permutation test
OTU:	Operational taxonomic unit
pHF-OS:	Partially hydrolyzed protein infant formula containing oligosaccharides
PRC:	Principal response curve
SCFA:	Short-chain fatty acid

METHODS

Study design and fecal sample selection

This study arises from a double-blind, randomized, controlled, parallel-group nutritional intervention trial in infants with a parental history of allergic disease conducted in 10 specialist pediatric centers in Australia, Singapore, England, and Ireland from April 2006 to March 2011, as described elsewhere.[10] In total, 1047 infants were recruited and consisted of 3 groups of participants. The breast-fed reference group consisted of exclusively breast-fed infants for the first 18 weeks of life. Participants who chose to stop breast-feeding or to supplement with formula before 18 weeks of age were randomized to receive either partially hydrolyzed whey protein–dominant infant formula containing short-chain galacto-oligosaccharides and long-chain fructo-oligosaccharides (9:1; 0.68 g/100 mL) and pectin-derived acidic oligosaccharides (0.12 g/100 mL) or standard cow's milk formula (control) up to 26 weeks of age. Information on trial ethics approvals, monitoring, and regulatory compliance are summarized in the Online Repository at www.isrctn.com (PATCH trial; study registration: ISRCTN65195597; February 14, 2006).

Fecal samples for microbial analysis were selected from infants who met the following criteria. Infants (I) were randomized to investigational formula before 4 weeks of age, or were part of the breast-fed reference group (n = 942 infants), (II) were vaginally born (normal or instrumental delivery, n = 673 infants), and (III) had stool specimens available at 4 and 26 weeks of age (n = 324 infants). A subset of these infants was randomly selected to obtain 60 infants for each formula group and 30 breast-fed reference infants (n = 150 infants).

Sample collection and preparation

Infants' fecal samples were collected by the parents into 10-mL stool containers (Greiner Bio-One, Kremsmünster, Austria), immediately frozen (−12°C to −20°C), and transported within 3 months to the hospital. On arrival at the hospital and before evaluation at the laboratory, samples were kept and transported at −80°C. Frozen stool samples were defrosted on ice, and stool pH was measured by using a pH meter equipped with a glass-body pH electrode (Mettler-Toledo, Columbus, Ohio). Fecal samples were 10-fold diluted in PBS buffer (150 mmol/L NaCl, 10 mmol/L Na_2HPO4, and 20 mmol/L NaH_2PO_4, pH 7.4), and 5 to 10 glass beads (3 mm in diameter) were added to homogenize the sample by means of vortexing for 3 minutes, followed by centrifugation (300*g* for 1 minute). Several 1-mL portions of supernatant were stored at −80°C for downstream processing.

16S rRNA gene sequencing and bioinformatics

Fecal suspensions of 200 μL were mixed with 450 μL of DNA extraction buffer (100 mmol/L Tris-HCl and 40 mmol/L EDTA, pH 9.0) and 50 μL of 10% SDS. Phenol-chloroform extractions combined with beat beating were performed as described by Matsuki et al,[11] except that extracted DNA was resuspended in 0.1 mL of TE (10 mmol/L Tris-HCl and 1 mmol/L EDTA, pH 8.0).

The V3 to V5 regions of the 16S rRNA gene were amplified by using the forward primer 357F and a "bifidobacteria-optimized" reverse primer, 926Rb.[12] 16S rDNA PCR was carried out in quadruplicates and pyrosequenced in three 454 GS FLX (Roche, Branford, Conn) runs, as previously

described.[12] Raw pyrosequencing data for all samples have been deposited in the Sequence Read Archive under accession number PRJEB19801.

The Quantitative Insights Into Microbial Ecology, version 1.5.0, package was used to analyze sequence data,[13] as previously described,[12] except that alignments were carried out with the SILVA rRNA database (SSU_REF108).[14]

Fecal SCFAs and lactic acid

Fecal suspensions were thawed on ice and centrifuged for 10 minutes at 14,000*g*. Then 350 μL of supernatant was inactivated by heating for 10 minutes at 100°C, followed by centrifugation. A portion of the supernatant was used to quantitatively determine the SCFAs acetic, propionic, n-butyric, isobutyric, and n-valeric acid by using gas chromatography, as described previously.[15] Another portion of the supernatant was used to enzymatically analyze lactate levels by using a D-/L-lactic acid assay kit (Megazyme, Wicklow, Ireland).

Data handling and statistical analyses

Multivariate statistical analyses of 16S rRNA gene sequencing data (taxonomic data set) and levels of SCFAs and lactate (metabolite data set) were performed through a combination of constrained ordination methods by using the Canoco 5 software[16] and differential abundance testing by using the R-package MetagenomeSeq.[17]

Counts of operational taxonomic units (OTUs) were aggregated at the genus level (resulting in 142 genera) and normalized by total sum scaling and $\log_2$-transformed. Genera present in less than 10 samples were discarded to remove sparse taxa, resulting in 58 features that were used as input for statistical analyses.

Monte Carlo permutation tests (MCPTs) were used to evaluate statistical significance ($P \leq .05$) of explanatory variables (constraints) in the ordination analyses performed. The Benjamini-Hochberg false discovery rate was used to account for multiple comparisons,[18] with significance for adjusted *P* values at .05, except for taxonomic features associated with eczema development, for which significance was considered at .1.

Canonical correspondence analysis was applied on the taxonomic data set because the unimodal model was found to best fit the relative abundances of the bacterial genera.[19] Forward selection was applied to identify sample covariates that best explained the variation in microbial taxonomic composition,[20] and these were subsequently used in all multivariate models and comparisons performed, either as explanatory variables or as covariates. Temporal changes of bacterial metabolites were assessed by using a linear ordination method known as principal response curves (PRCs)[21] because the linear model was found to best fit the bacterial metabolite data set.

Univariate data analyses were performed with GraphPad Prism software, version 6.02, for Windows (GraphPad Software, La Jolla, Calif), applying the Mann-Whitney test for 2-group comparisons and 1-way ANOVA with the Bonferroni multiple comparisons test for 3-group comparisons, with a significance of .05.

RESULTS

Effects of pHF-OS on microbial richness and diversity

In total, 12 of the 150 selected infants did not have stool specimens available at 4 and 26 weeks of age. The characteristics of the remaining 138 participants are summarized in Table I. Pyrosequencing was performed on stool specimens collected at 4 and 26 weeks of age, representing specimens taken after randomization and at the end of the intervention period, respectively. The majority of randomized infants were already receiving formula before 4 weeks of age (Table I). A total of 8 specimens collected at 4 weeks of age had insufficient amounts for preparation. The remaining 268 samples were successfully sequenced, with a mean sequence depth of 6211 reads and a mean read length of 521 nucleotides (see Table E1 in this article's Online Repository at www.jacionline.org).

TABLE I. Characteristics of the study population

	Control subjects (n = 57)	Infants receiving pHF-OS (n = 51)	Breast-fed infants (n = 30)
Started formula before 4 wk	54 (95%)	48 (84%)	NA
Male sex	23 (40%)	30 (59%)	18 (60%)
Birth weight (g)	3317 (502)	3461 (442)	3606 (393)
Both parents allergic	11 (19%)	6 (12%)	11 (37%)
Only mother allergic	33 (58%)	29 (57%)	11 (37%)
Only father allergic	13 (23%)	16 (31%)	8 (27%)
Pet at home	11 (19%)	22 (43%)	7 (23%)
At least 1 sibling	28 (49%)	27 (53%)	22 (73%)
Vaginal delivery	46 (81%)	41 (80%)	25 (83%)
Instrumental delivery	11 (19%)	10 (20%)	5 (17%)
White ethnicity	16 (28%)	28 (55%)	25 (83%)
Asian ethnicity	35 (61%)	21 (41%)	2 (7%)
Other ethnicity	6 (12%)	2 (4%)	3 (10%)
Australia	13 (23%)	14 (27%)	13 (43%)
Ireland	4 (7%)	10 (20%)	5 (17%)
Singapore	35 (61%)	21 (41%)	2 (7%)
United Kingdom	5 (9%)	6 (12%)	10 (33%)
Eczema by 12 mo	15 (26%)	17 (33%)	12 (40%)
Eczema by 18 mo	18 (32%)	20 (39%)	14 (47%)
Antibiotics by 26 wk	6 (11%)	12 (24%)	3 (10%)

Continuous data are presented as means (SDs).
NA, Not applicable.

Microbial richness and diversity of the different feeding groups was assessed at a sequence depth of 1636 reads. Richness was expressed as the number of unique OTUs (observed species), and diversity was assessed by using a measure of phylogenetic diversity (PD whole tree metric). This measure takes both the number of unique OTUs and their phylogenetic distance into account.[22] No differences between the randomization groups were observed, but the breast-fed reference group showed a significantly lower richness and diversity at 26 weeks when compared with all formula-fed infants combined ($P < .01$ and $P < .05$, 1-way ANOVA, respectively; Fig 1).

pHF-OS modulates the microbial composition closer to that of breast-fed infants

The majority of OTUs were assigned to 4 phyla, namely Actinobacteria (65.0%), Firmicutes (24.8%), Proteobacteria (8.6%), and Bacteroidetes (1.5%, see Table E2 in this article's Online Repository at www.jacionline.org). The weighted UniFrac distance metric, which is based on phylogenetic distances and relative abundances of bacterial taxa in a pairwise comparison of samples,[23] was used to assess the overall (dis)similarity in bacterial composition when comparing infants receiving pHF-OS with the control group and their respective distances with the breast-fed reference group. Gut microbial compositions of infants receiving pHF-OS were found to be significantly more similar to those of breast-fed infants than the compositions of control group to breast-fed infants at 26 weeks ($P < .0001$, 1-way ANOVA; Fig 2, *A*).

Canonical correspondence analysis was combined with forward selection of variables to identify sample covariates that best

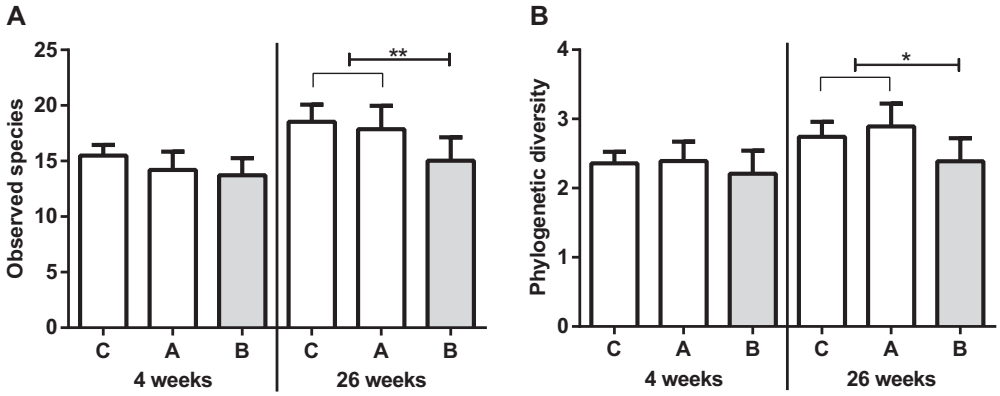

FIG 1. Bar plots (means with 95% CIs) summarizing microbial intestinal richness **(A)** and phylogenetic diversity **(B)** at 4 and 26 weeks of age in breast-fed infants *(B)* and infants receiving pHF-OS *(A)* or control formula *(C)*. Comparisons were done at a sequencing depth of 1636 reads per sample. Statistics were performed by using 1-way ANOVA with the Bonferroni multiple comparison correction. *Adjusted $P < .05$ and **adjusted $P < .01$.

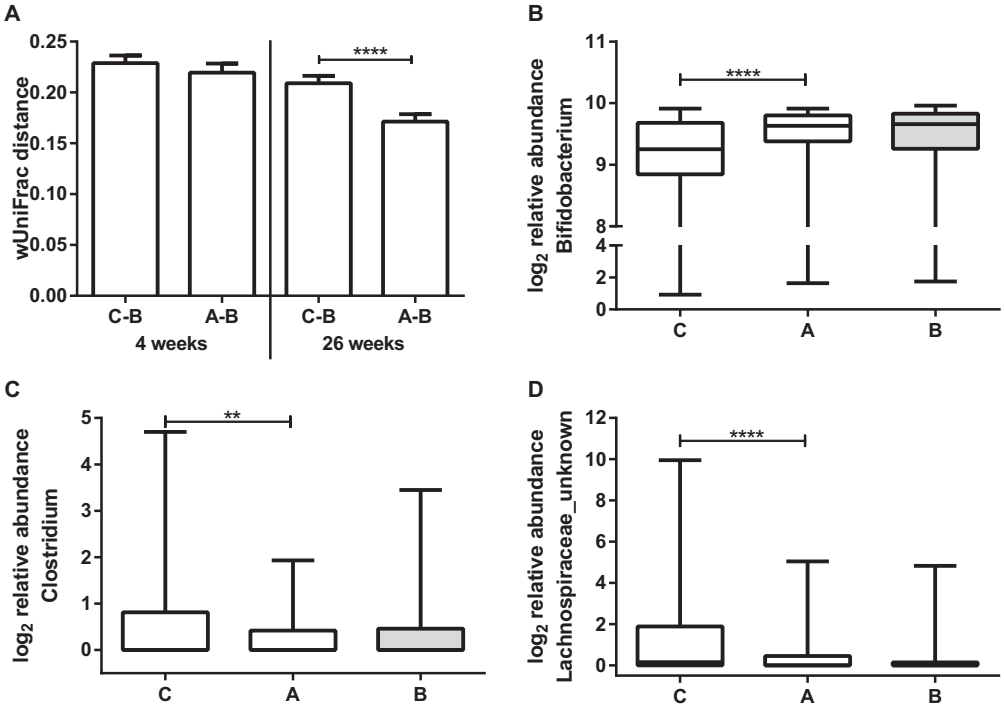

FIG 2. A, Bar plots (means with 95% CIs) summarizing weighted UniFrac distances at 4 and 26 weeks of age in infants receiving pHF-OS or control formula relative to breast-fed infants (*A-B* vs *C-B*, respectively). Distances are measured on a scale from 0 to 1, with 0 meaning 100% identical and 1 meaning 0% identical gut microbial composition. Statistical comparison of weighted UniFrac was performed by using a 1-way ANOVA with the Bonferroni multiple comparison correction. ****Adjusted $P < .0001$. **B-D,** Box plots summarizing $\log_2$-transformed levels of differential abundant genera present in more than 15% of samples and a mean relative abundance of greater than 0.1% when comparing pHF-OS with control formula at 26 weeks, namely *Bifidobacterium* species (Fig 2, *B*), *Clostridium* species (Fig 2, *C*), and an unassigned genus of Lachnospiraceae (Fig 2, *D*). Differential abundances were computed by using MetagenomeSeq, with correction for effects of ethnicity and having siblings and adjustment of significance values for multiple comparisons by using the Benjamini-Hochberg false discovery rate. **Adjusted $P < .01$ and ****adjusted $P < .0001$.

explained the taxonomic composition of the fecal samples.[20] This analysis identified time (age in weeks), ethnicity (Asian, white, or other), feeding group (control, pH-OS, or breast-fed), and having siblings (yes/no) as the factors significantly explaining the taxonomic variation (adjusted $P < .05$, see Table E3 in this article's Online Repository at www.jacionline.org).

MetagenomeSeq[17] was subsequently used to assess which bacterial taxa accounted for the differences observed in weighted UniFrac at 26 weeks while correcting for the covariates identified. Infants receiving pHF-OS were discriminated from control subjects by increased levels of *Bifidobacterium* species and decreased levels of *Clostridium*

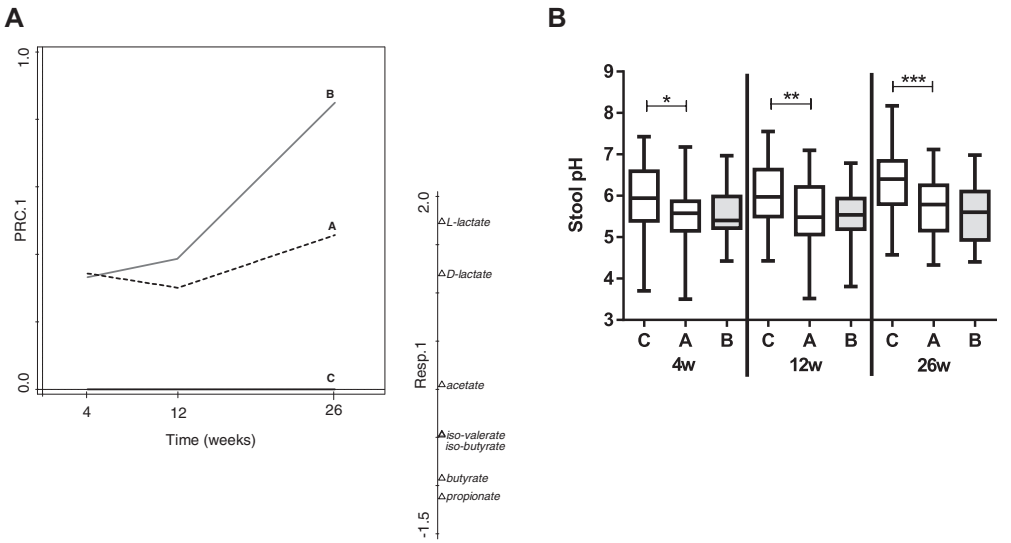

FIG 3. A, PRCs showing changes in bacterial metabolites (levels of SCFAs and lactic acid) across time and the interaction with the different feeding groups. The *horizontal axis* represents time, and the *vertical axis* represents PRC score values. Fecal metabolite composition of infants receiving control formula *(C)* was used as a reference level and has zero PRC values; therefore its curve is over the *horizontal axis*. Changes for infants being breast-fed *(B)* or receiving pHF-OS *(A)* are shown as response curves relative to this reference. Metabolite response scores are shown on the separate vertical (1-dimensional) plot. The multiple of the PRC score with the response score provides a quantitative interpretation, as well as the direction of change at respective time points (4, 12, and 26 weeks). Significance of the interaction corrected for covariates was tested by using MCPT ($P = .002$, 499 permutations). **B,** Box plots summarizing stool pH at 4, 12, and 26 weeks of age for the different feeding groups. Statistical comparison was performed by using 1-way ANOVA with the Bonferroni multiple comparison correction comparing pHF-OS with control formula. *Adjusted $P < .05$, **adjusted $P < .01$, and ***adjusted $P < .001$.

species and an unassigned genus of Lachnospiraceae (Fig 2, *B-D*).

pHF-OS modulates the microbial activity closer to that of breast-fed infants

PRCs were used to assess temporal effects of the feeding groups on fecal composition of SCFAs and lactate in stool specimens collected at 4, 12, and 26 weeks, respectively. The interaction of feeding group with time, corrected for ethnicity and having siblings, was significant on the first constrained axis (explained variation = 80.3%, MCPT: $P = .002$ with 499 permutations). Both the metabolite composition of breast-fed infants and that of infants receiving pHF-OS were characterized by increased proportions of D- and L-lactate at 4, 12, and 26 weeks of age and decreased proportions of propionate, butyrate, and branched-chain SCFA (isobutyrate and isovalerate) in contrast to the control group (Fig 3, *A*). Moreover, infants receiving pHF-OS were found to have significantly decreased stool pH at all time points compared with infants receiving control formula, with ranges similar to those observed for breast-fed infants (Fig 3, *B*).

Aberrant temporal dynamics in infants with eczema in the first 18 months of life

In total, 52 of the 138 infants had eczema by 18 months of age (Table I). No differences in bacterial richness or diversity were observed at 4 or 26 weeks of age when comparing eczematous with noneczematous infants (see Fig E1 in this article's Online Repository at www.jacionline.org).

PRCs were used to investigate the fecal metabolite composition over time for infants with eczema compared with those without eczema while correcting for the covariates identified (ethnicity, feeding group, and having siblings). Significant temporal differences on the first constrained axis produced (explained variation = 84.4%, MCPT: $P = .034$ with 499 permutations) were observed for the interaction (Fig 4, *A*). Differential dynamics were most pronounced from 12 to 26 weeks of age. Infants with eczema were characterized by decreased levels of both isomers of lactate and increased levels of propionate and butyrate at 12 weeks. This pattern was reversed at 26 weeks of age, with infants having eczema showing increased levels of lactate and decreased levels of propionate and butyrate. No significant differences were observed in stool pH (Fig 4, *B*).

Decreased microbial conversion of lactic acid into butyrate in infants with eczema

MetagenomeSeq was used to assess differential abundances of bacterial taxa over time in infants with and without eczema and which of those taxa were differential over time between the 2 groups. The analyses were corrected for the identified covariates (Fig 5, *A*). Decreases of *Staphylococcus* and *Streptococcus* species over time were observed in both healthy infants and infants with eczema. Two genera of Lachnospiraceae (*Blautia* species and an unassigned genus designated as "other") and Erysipelotrichaceae were found to increase over time in both groups. The genus *Bifidobacterium* was found to increase over time in healthy infants and to decrease in infants with eczema; however, this pattern was not significantly different when comparing the 2

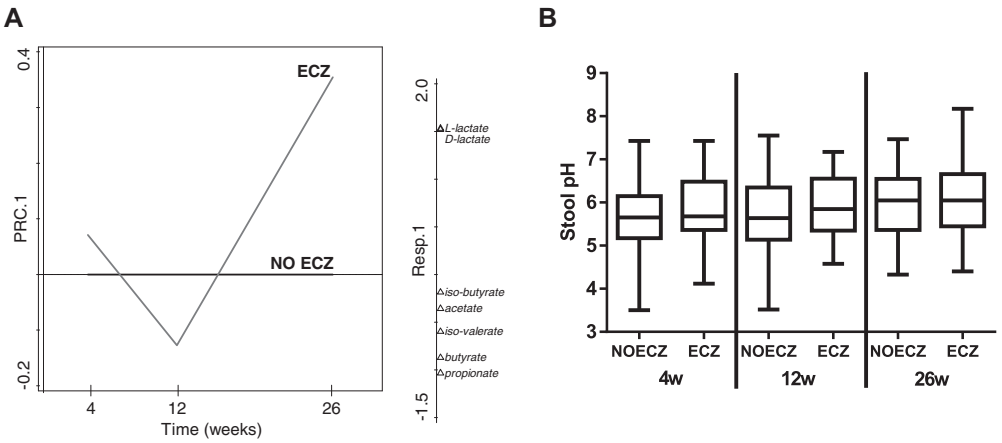

FIG 4. A, PRCs showing changes in bacterial metabolites (levels of SCFAs and lactic acid) across time and interaction with eczema development. The *horizontal axis* represents time, and the *vertical axis* represents PRC score values. The fecal metabolite composition of infants without eczema *(NO ECZ)* were used as the reference level and has zero PRC values. Change for infants having eczema in the first 18 months of life *(ECZ)* is shown as a response curve relative to this reference. Metabolite response scores are shown on the separate vertical (1-dimensional) plot. Significance of the interaction corrected for effects of feeding group, ethnicity, and having siblings was tested by using MCPT ($P = .034$, 499 permutations). **B,** Box plots summarizing stool pH at 4, 12, and 26 weeks of age in infants with eczema versus healthy infants. Statistical comparison was performed by using a 1-way ANOVA with the Bonferroni multiple comparison correction (not significantly different).

groups (adjusted $P = .115$). Differential patterns over time were observed for 2 genera of Enterobacteriaceae, namely *Enterobacter* species (adjusted $P < .001$) and an unassigned genus of Enterobacteriaceae (adjusted $P < .1$). These differences were mostly driven by increased levels of these genera in healthy infants at 4 weeks of age and a subsequent decrease over time. This pattern was less pronounced in infants with eczema (see Fig E2, *A* and *B*, in this article's Online Repository at www.jacionline.org). Differential development over time was also observed for *Parabacteroides* species (adjusted $P < .0001$), with a decrease in healthy infants and an increase in infants with eczema (see Fig E2, *C*). Furthermore, the increases over time observed for *Eubacterium* (adjusted $P < .05$) and *Anaerostipes* (adjusted $P < .1$) species were stronger in healthy infants compared with those in infants with eczema (see Fig E2, *D* and *E*, respectively). Both genera are associated with a specialist group of microbes known to convert lactate together with acetate into mainly butyrate, hence referred to as lactate-utilizing and butyrate-producing bacteria (LUBs).[24,25] Moreover, at 26 weeks of age, decreased levels of both D- and L-lactate (Fig 5, *B* and *C*) and increased levels of butyrate (Fig 5, *D*) were observed in healthy infants compared with those in infants with eczema.

DISCUSSION

In this study we found that a partially hydrolyzed protein formula supplemented with a specific oligosaccharide mixture (pHF-OS) modulates the developing gut microbiota of infants toward a pattern closer to that of breast-fed infants both in bacterial taxonomic composition and in metabolite composition. Infants receiving pHF-OS from 4 to 26 weeks of age showed increased relative abundances of the genus *Bifidobacterium*, which was contrasted by decreases of *Clostridium* species and an unassigned genus of Lachnospiraceae, when compared with infants receiving standard cow's milk formula. These modulations were reflected in marked differences in gut physiology characterized by lower stool pH, increased proportions of lactate, and decreased proportions of propionate, butyrate, isobutyrate, and isovalerate.

Furthermore, we found that infants with eczema in the first 18 months of life showed aberrant gut microbiota development in the first 26 weeks of life with significant temporal differences of the genus *Parabacteroides* and 2 genera of Enterobacteriaceae. These were found to decrease over time in healthy infants, a pattern that was reversed or less evident in infants with eczema. Additionally, infants with eczema showed a lower establishment over time of LUBs, namely *Eubacterium* and *Anaerostipes* species, supported by significantly increased fecal concentrations of lactate and decreased concentrations of butyrate at 26 weeks of age, a pattern that was independent of feeding group, ethnicity, or having siblings.

It is of critical importance to consider the dynamic nature of the infant gut microbiota and the environmental factors that influence its assembly to assess modulatory effects of nutrition and the role of specific microbes in eczema development.[26] Hence we excluded infants born by means of cesarean section to eliminate its confounding effects.[27] Moreover, multivariate comparisons were controlled for sample covariates that best explained the fecal compositional variation next to time (age in weeks) and feeding group, which were ethnicity and having siblings.

Other studies investigating the gut microbiota in relation to allergy development typically reported differences at specific points in time, especially in the first weeks of life, with most commonly reduced diversity and richness associated but often no specific microbes being identified.[2] We did not observe differences in diversity or richness but rather identified differences in the colonization patterns of infants with eczema compared with those without eczema by 18 months of age.

Some of these patterns were driven by early differences at 4 weeks of age, like the increased levels of 2 genera within the family of Enterobacteriaceae in subsequent healthy infants,

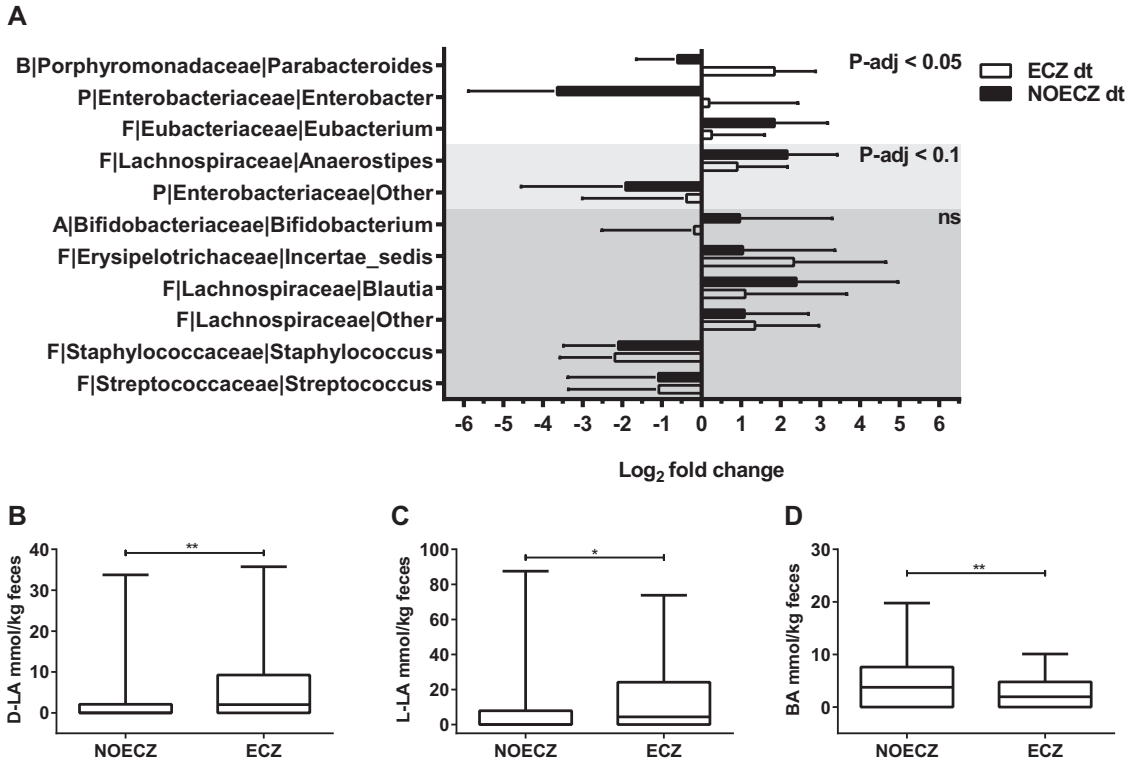

FIG 5. A, Plot with bacterial taxa that change over time (*dt* = change from 4 to 26 weeks of age) in healthy infants and infants with eczema, as well as taxa that are differential over time in infants with eczema compared with healthy infants. Data are shown as fold changes with SDs of $\log_2$-transformed data. Taxa are detailed at the phylum (*B*, Bacteroidetes; *F*, Firmicutes; and *P*, Proteobacteria), family, and genus levels, respectively. The contrasts were computed by using MetagenomeSeq and corrected for the effects of feeding group, ethnicity, and having siblings. Adjustment of significance values for multiple comparisons was done by using the Benjamini-Hochberg false discovery rate. Differential taxa with an adjusted *P* value of less than .05 for all contrasts if present in more than 15% of the samples and a mean abundance of greater than 0.1% are shown. Taxa plotted on the *dark gray background* showed no significant *(ns)* temporal differences when comparing healthy infants with eczematous infants. Taxa plotted on light gray and *white backgrounds* were different, with adjusted *P* values of less than .1 and less than .05, respectively. **B,** Fecal concentration of D-lactic acid *(D-LA)* at 26 weeks in healthy infants *(NOECZ)* compared with infants with eczema *(ECZ)*. **C,** Fecal concentration of L-lactic acid *(L-LA)* at 26 weeks of age in healthy subjects versus patients with eczema. **D,** Fecal concentration of butyrate *(BA)* at age 26 weeks in healthy subjects versus patients with eczema. Significance was tested with a Mann-Whitney 2-group comparison: **P* < .05 and ***P* < .001.

which was followed by sharp decreases over time. Facultative anaerobic bacteria, such as the Enterobacteriaceae, are typically high in the early colonization process before replacement with anaerobic bacteria. This pattern being less evident in infants with eczema might indicate a reduced immune stimulus from species within this family. These include several potential pathogens that might be needed for adequate development of the adaptive immune system. Indeed, a recent study by West et al[28] associated low abundance of Enterobacteriaceae at 1 month, with an exaggerated immune response at 6 months. This deviating pattern was confirmed in infants subsequently having allergy.[28-30]

It is known and was confirmed in this study that breast-fed infants have a lower stool pH compared with formula-fed infants, increased amounts of colonic acetate and lactate, and decreased amounts of propionate and butyrate. This reflects the dominance of *Bifidobacterium* species that produce acetate and L-lactate.[31] This specific colonic environment, also observed for infants receiving pHF-OS, is known to form an effective chemical barrier against potential pathogens[32,33] and is thought to contribute to the protective effects of breast-feeding against infections.[34,35]

We hypothesize that this typical infant-type colonic environment might also be crucial for the establishment of LUBs, such as *Eubacterium* and *Anaerostipes* species, which were found to be poorly established at 26 weeks of age in infants with eczema. Indeed, these infants showed first decreased levels of lactate at 12 weeks, followed by increased levels of lactate and decreased levels of butyrate at 26 weeks of age. This might indicate the importance of LUBs in guiding the transition around the weaning period (between 4-6 months of age) from a lactate- and acetate-rich environment toward a more adult-like butyrogenic milieu.[2,36] Moreover, lactate consumption might contribute to the development of a stable and healthy microbial ecosystem.[37] Indeed, lactate, although commonly detected in infant feces, is undetectable in healthy adults,[38] whereas accumulation of colonic lactate in adults has been associated with ulcerative colitis.[39,40]

The weaning period also exposes the infant to an increasing level of antigens from the diet, which requires the immune system

to adequately respond to these harmless substances. A study investigating the correlation of specific microbial signatures with the severity of eczema in 6-month-old infants found an inverse correlation with the levels of butyrate-producing bacteria and suggested a role in the observed alleviation of symptoms.[41] The role of SCFAs in the host, especially butyrate, has received increased interest because of accumulating evidence from murine studies showing their regulatory effects on host immunity, including anti-inflammatory and anti-allergic effects.[42-45] Therefore the establishment of butyrate-producing bacteria around 26 weeks of age, possibly specifically those cross-feeding on lactate and acetate, might prove important for establishing and maintaining homeostasis with our immune system during this critical stage of development.

In conclusion, this study confirms the effect of early-life nutrition on establishment of the infant gut microbiota. Moreover, it indicates a potential link between the activity of the microbiota and the expression of eczema in early life. It emphasizes the importance of the microbial succession of species and metabolite cross-feeding to develop a gut physiology that supports gut development and also supports development of normal immune responses to environmental triggers. These observations could aid in the development of optimal nutritional strategies to support the timely gut colonization of keystone species in the gradually diversifying infant gut.

We thank all infants and their families who took part in the study and all the PATCH investigators for their collaboration in this study.

Key messages

- **A partially hydrolyzed protein infant formula supplemented with specific prebiotic oligosaccharides modulates the fecal microbiota of high-risk infants for allergy closer to that of breast-fed infants, both in taxonomic composition and in metabolite composition.**
- **Infants with eczema in the first 18 months showed discordant temporal development of bacterial taxa and metabolites in the first 26 weeks of life. The identified patterns might lead to the development of both preventive and therapeutic targets for eczema and other manifestations of allergy.**

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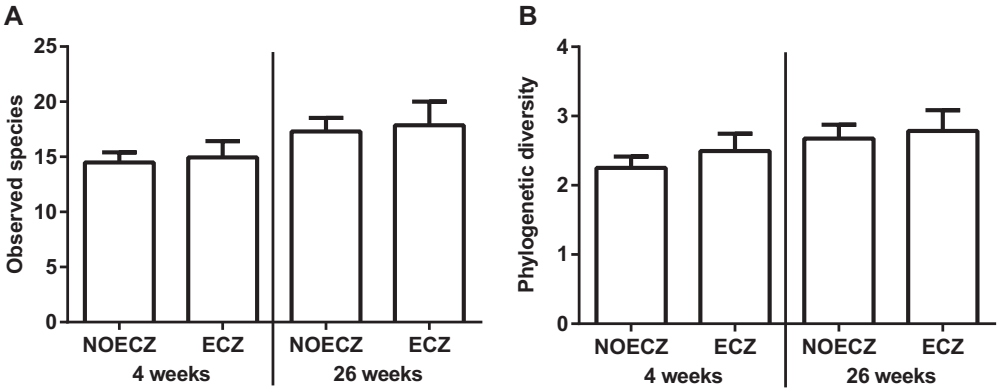

FIG E1. Bar plots (means with 95% CIs) summarizing the microbial intestinal richness **(A)** and phylogenetic diversity **(B)** at 4 and 26 weeks of age in infants with eczema *(ECZ)* compared with infants without eczema *(NOECZ)*. Comparisons were done at a sequencing depth of 1636 reads per sample. Statistics were performed by using a 1-way ANOVA with the Bonferroni multiple comparison correction (not significantly different).

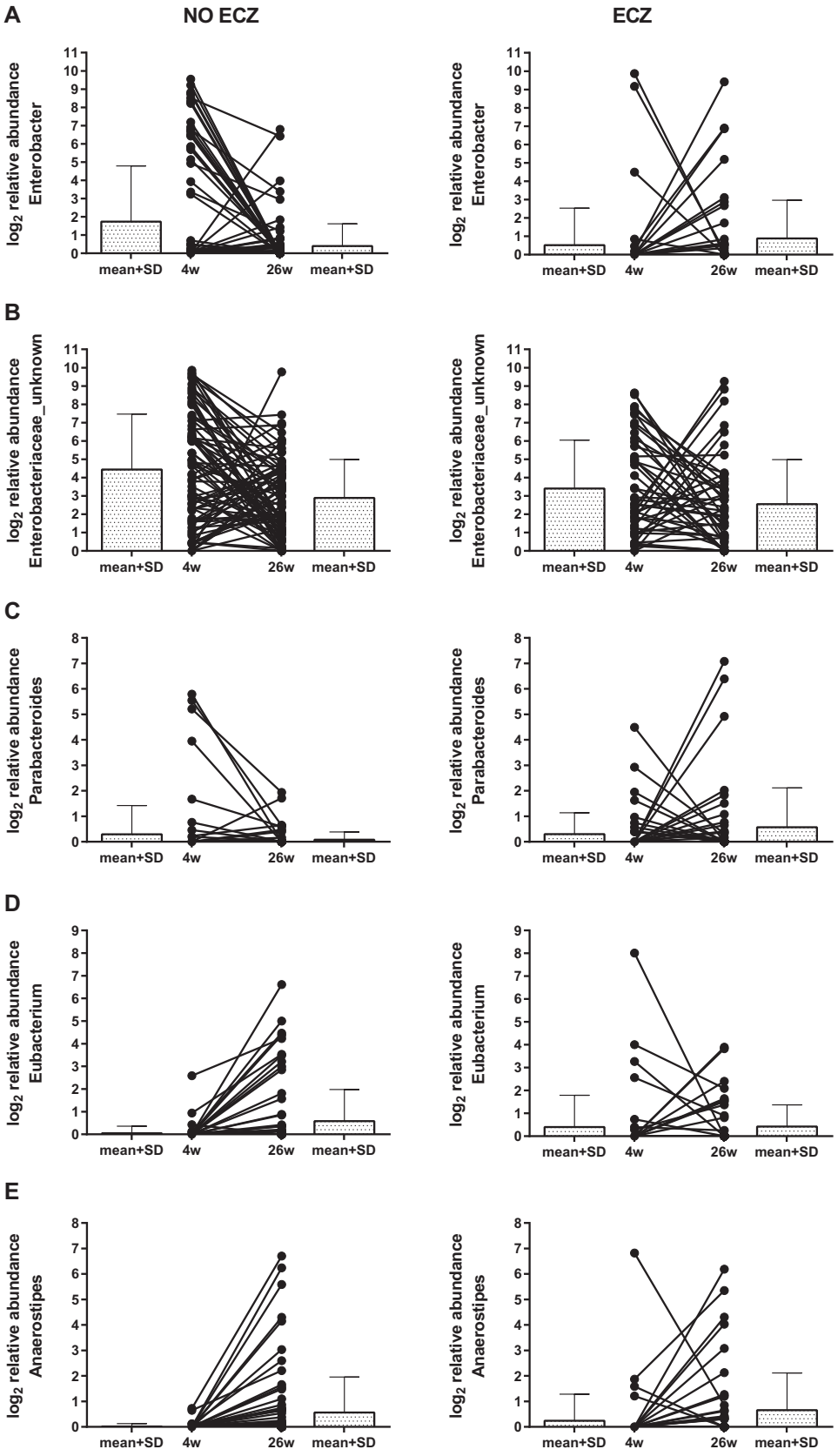

FIG E2. Taxa identified with MetagenomeSeq that show differential relative abundances over time when comparing healthy infants with infants with eczema (adjusted $P < .1$). Levels for sample pairs are shown as $\log_2$-transformed data, as well as means and SDs at 4 and 26 weeks of age, and are shown for *Enterobacter* **(A)**, an unassigned genus of Enterobacteriaceae **(B)**, *Parabacteroides* **(C)**, *Eubacterium* **(D)**, and Anaerostipes **(E)** species, respectively. *Left panels* show data for infants without eczema (*NO ECZ*; n = 82 sample pairs), and *right panels* show data for infants with eczema (*ECZ*; n = 48 sample pairs), respectively.

TABLE E1. Sample size and sequence details for sequences passing quality filters summarized per feeding group

Feeding group	Age (wk)	No. of subjects	Sum of reads	Average sequence depth	SD of sequence depth
Control subjects	4	55	326,169	5,930	3,300
	26	57	345,935	6,069	2,959
Infants receiving pHF-OS	4	49	305,530	6,235	4,459
	26	51	299,308	5,869	4,113
Breast-fed infants	4	26	166,838	6,417	3,950
	26	30	220,892	7,363	4,014
Total		268	1,664,672	6,211	3,767

TABLE E2. Summary of gut microbial compositions of breast-fed infants *(B)*, infants receiving pHF-OS *(A)*, and control subjects *(C)* at 4 and 26 weeks of age

Taxa	4 wk			26 wk			
Phylum/order/family	**C**	**A**	**B**	**C**	**A**	**B**	**Grand total**
Total Actinobacteria	**55.28 (32.66)%**	**61.83 (31.56)%**	**69.66 (35.33)%**	**63.01 (28.43)%**	**73.25 (27.08)%**	**73.12 (28.76)%**	**64.92 (31.21)%**
A/Bifidobacteriales/ Bifidobacteriaceae	51.39 (32.25)%	60.18 (31.18)%	68.95 (35.13)%	59.99 (27.59)%	71.02 (26.73)%	72.38 (28.52)%	62.6 (30.98)%
A/Coriobacteriales/ Coriobacteriaceae	3.56 (9.53)%	1.19 (3.14)%	0.34 (1.01)%	2.99 (6.92)%	2.2 (4.13)%	0.65 (1.63)%	2.12 (6.01)%
A/Propionibacteriales/ Propionibacteriaceae	0.28 (0.86)%	0.4 (1.24)%	0.26 (1.01)%	0.01 (0.04)%	0.01 (0.05)%	0 (0)%	0.15 (0.74)%
A/other	0.06 (0.09)%	0.06 (0.27)%	0.11 (0.23)%	0.02 (0.04)%	0.02 (0.05)%	0.08 (0.21)%	0.05 (0.16)%
Total Bacteroidetes	**1.88 (8.01)%**	**1.15 (3.65)%**	**3.06 (5.45)%**	**0.33 (1.42)%**	**1.53 (4.49)%**	**1.94 (5.82)%**	**1.49 (5.26)%**
B/Bacteroidales/ Bacteroidaceae	1.77 (7.9)%	1 (3.17)%	2.45 (5.21)%	0.31 (1.39)%	1.17 (3.48)%	1.44 (3.5)%	1.25 (4.71)%
B/Bacteroidales/ Porphyromonadaceae	0.10 (0.63)%	0.14 (0.83)%	0.22 (0.73)%	0.01 (0.04)%	0.31 (1.58)%	0.48 (2.45)%	0.18 (1.2)%
B/other	0.01 (0.02)%	0.01 (0.03)%	0.39 (1.9)%	0.01 (0.04)%	0.05 (0.24)%	0.02 (0.04)%	0.06 (0.63)%
Total Firmicutes	**31.09 (24.75)%**	**23.34 (21.26)%**	**8.63 (8.59)%**	**32.04 (24.86)%**	**21.85 (22.06)%**	**21.16 (25.58)%**	**24.81 (23.72)%**
F/Bacillales/ Staphylococcaceae	0.52 (1.44)%	0.44 (1.17)%	0.72 (1.57)%	0.03 (0.06)%	0.03 (0.14)%	0.02 (0.06)%	0.27 (1.00)%
F/Lactobacillales/ Enterococcaceae	8.69 (13.3)%	1.19 (2.53)%	0.6 (2.02)%	4.84 (8.26)%	4.11 (5.92)%	2.38 (4.33)%	4.18 (8.36)%
F/Lactobacillales/ Lactobacillaceae	2.54 (7.67)%	2.82 (5.67)%	0.78 (1.69)%	2.01 (5.27)%	1.05 (2.91)%	1.31 (2.94)%	1.87 (5.22)%
F/Lactobacillales/ Streptococcaceae	12.68 (16.91)%	12.42 (16.31)%	3.05 (4.92)%	8.85 (16.43)%	5.34 (8.99)%	3.94 (10.11)%	8.44 (14.37)%
F/Clostridiales/ Clostridiaceae	0.29 (1.21)%	0.05 (0.15)%	0.11 (0.51)%	0.17 (0.46)%	0.04 (0.08)%	0.07 (0.19)%	0.13 (0.64)%
F/Clostridiales/ Eubacteriaceae	0.06 (0.25)%	0 (0.01)%	0 (0.01)%	0.34 (1.38)%	0.12 (0.38)%	0.08 (0.28)%	0.12 (0.69)%
F/Clostridiales/ Lachnospiraceae	2.74 (8.59)%	5.7 (15.79)%	2.59 (5.52)%	13.56 (22.46)%	7.87 (15.07)%	8.5 (20.01)%	7.17 (16.55)%
F/Clostridiales/ Peptostreptococcaceae	0.08 (0.29)%	0.00 (0.01)%	0.00 (0.00)%	0.14 (0.35)%	0.08 (0.18)%	0.20 (0.66)%	0.09 (0.33)%
F/Clostridiales/ Ruminococcaceae	0.01 (0.05)%	0.00 (0.00)%	0.01 (0.03)%	0.15 (0.96)%	0.34 (2.14)%	0.13 (0.56)%	0.11 (1.05)%
F/Clostridiales/ Veillonellaceae	0.87 (2.23)%	0.05 (0.14)%	0.11 (0.29)%	0.06 (0.19)%	0.18 (0.53)%	0.41 (1.33)%	0.30 (1.2)%
F/Erysipelotrichales/ Erysipelotrichaceae	2.51 (9.88)%	0.63 (1.73)%	0.63 (2.1)%	1.83 (3.63)%	2.65 (7.78)%	4.08 (10.63)%	2.06 (7.07)%
F|other	0.07 (0.18)%	0.03 (0.09)%	0.04 (0.05)%	0.05 (0.05)%	0.03 (0.07)%	0.05 (0.12)%	0.05 (0.11)%
Total Proteobacteria	**11.72 (19.76)%**	**13.67 (22)%**	**18.64 (30.75)%**	**4.07 (13.77)%**	**3.13 (8.88)%**	**3.78 (11.22)%**	**8.61 (19.03)%**
P/Enterobacteriales/ Enterobacteriaceae	11.54 (19.24)%	13.61 (21.96)%	18.5 (30.7)%	4 (13.74)%	3.11 (8.86)%	3.53 (11.08)%	8.5 (18.87)%
P/other	0.18 (0.83)%	0.06 (0.12)%	0.14 (0.32)%	0.07 (0.33)%	0.02 (0.05)%	0.25 (0.83)%	0.11 (0.52)%
V/Verrucomicrobiales/ Verrucomicrobiaceae	**0.01 (0.09)%**	**0.01 (0.07)%**	**0.00 (0.00)%**	**0.54 (2.74)%**	**0.23 (0.88)%**	**0.00 (0.00)%**	**0.16 (1.35)%**
Other	**0.02 (0.06)%**	**0.01 (0.02)%**	**0.01 (0.02)%**	**0.01 (0.02)%**	**0.01 (0.02)%**	**0.01 (0.01)%**	**0.01 (0.03)%**

Relative abundances with SDs in percentages for taxa present in more than 15% of the samples and average relative abundances of greater than 0.1% at the bacterial family-level. Taxa of lower abundance are grouped as "other." Phylum-levels are indicated as follows: *A*, Actinobacteria; *B*, Bacteroidetes; *F*, Firmicutes; *P*, Proteobacteria; *V*, Verrucomicrobia. Boldface text represents subtotals.

TABLE E3. Results of forward selection based on canonical correspondence analysis of fecal taxonomic compositions

Sample covariates (variables)	Explains (%)	*P* value	Adjusted *P* value
Age (4 wk, 26 wk)	**5.2**	**.002**	**.01733**
Ethnicity (Asian, white, other)	**3.7**	**.002**	**.0104**
Feeding group (control, pHF-OS, breast-fed)	**2.6**	**.002**	**.00867**
Siblings (yes, no)	**1.6**	**.002**	**.00867**
Antibiotics before 26 wk (yes, no)	1.2	.022	.08089
Birth weight (continuous data)	0.6	.028	.104
Sex (male, female)	0.8	.206	.44
Weaning before 18 wk (yes, no)	0.8	.226	.41947
Parental allergic history (mother, father, both)	1	.266	.43225

Sample covariates shown were included based on the percentage explained of the total taxonomic variation *(Explains [%])*. Inclusion of sample covariates was stopped to prevent overfitting of the ordination model, leading to identification of the 9 factors (and variables) listed in the table. The false discovery rate method for *P* value correction was used to identify the major covariates with an adjusted *P* value of less than .05 (in boldface) and shown alongside the uncorrected *P* value.