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Establishment of Intestinal Microbiota during Early Life: a Longitudinal, Explorative Study of a Large Cohort of Danish Infants

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Fecal samples were obtained from a cohort of 330 healthy Danish infants at 9, 18, and 36 months after birth, enabling characterization of interbacterial relationships by use of quantitative PCR targeting 31 selected bacterial 16S rRNA gene targets representing different phylogenetic levels. Nutritional parameters and measures of growth and body composition were determined and investigated in relation to the observed development in microbiota composition. We found that significant changes in the gut microbiota occurred, particularly from age 9 to 18 months, when cessation of breastfeeding and introduction of a complementary feeding induce replacement of a microbiota characterized by lactobacilli, bifidobacteria, and *Enterobacteriaceae* with a microbiota dominated by *Clostridium* spp. and *Bacteroides* spp. Classification of samples by a proxy enterotype based on the relative levels of *Bacteroides* spp. and *Prevotella* spp. showed that enterotype establishment occurs between 9 and 36 months. Thirty percent of the individuals shifted enterotype between 18 and 36 months. The composition of the microbiota was most pronouncedly influenced by the time of cessation of breastfeeding. From 9 to 18 months, a positive correlation was observed between the increase in body mass index and the increase of the short-chain-fatty-acid-producing clostridia, the *Clostridium leptum* group, and *Eubacterium hallii*. Considering previously established positive associations between rapid infant weight gain, early breastfeeding discontinuation, and later-life obesity, the corresponding microbial findings seen here warrant attention.

Establishment of the human intestinal microbiota during infancy is influenced by multiple factors, including delivery mode, sanitary conditions, administration of antibiotics to the infant or mother (1, 2), and level of breastfeeding (3). Breastfeeding has been shown to significantly increase the relative abundance of bifidobacteria and lactic acid bacteria, including lactobacilli and *Enterococcus* spp. (4, 5). The microbial composition within the first year of life is typically characterized by low species diversity and high instability (6–8). Nevertheless, a number of recent studies suggest that some of the bacteria that become part of the adult microbiota colonize the gut already during the first months of life (9, 10). A more complex, stable, and adult-like microbiota is established between 1 and 2 years after birth (11–13), the composition of which is believed to affect the risk of several lifestyle-related disorders, including obesity and type 2 diabetes (14, 15). Increased prevalence of childhood obesity is currently a major societal concern due to the high association with adult obesity (16, 17). It has also been proposed that obesity in adults may be related to the capacity of the gut microbiota to harvest energy through breakdown of indigestible polysaccharides (18, 19). Moreover, obesity and insulin resistance are often accompanied by a state of chronic low-grade inflammation (20). Since species-specific bacterial surface markers are involved in regulation of inflammation, differences in commensal bacterial composition between individuals may differentially predispose them to inflammation-induced diseases (21). Breastfed infants are leaner than formula-fed counterparts (22, 23) and display lower incidences of obesity, diabetes, and inflammatory bowel diseases later in life (24), and a link between diet, infant gut microbiota, obesity development, and inflammatory pathways has thus been suggested (25).

The discovery of the existence of so-called gut enterotypes in adult human subjects has received considerable attention, although final consensus on the number and characteristics of the enterotypes has not yet been achieved (26, 27). In this context, it has recently been proposed by us that adults can be grouped into two distinct groups based on the levels of *Bacteroides* spp. and particularly *Prevotella* spp., which are bimodally distributed in adult subjects and remain stable over a period of at least 6 months, and that these two genera can therefore serve as markers for the corresponding enterotypes (28). Each adult individual is estimated to harbor approximately 160 different high-abundance species, many of which are expected to be shared between individuals but present at very different levels in different individuals (29). Looking at the total pool of species present, earlier studies have suggested that as little as 1% may be shared with another individual (7, 30). Therefore, some redundancy of colonic bacterial processing would be expected to exist, and it has been demonstrated that within the intestinal metagenome, the phylogenetic variation is much more pronounced than the variation in functional capacities of the ecosystem (31). While interdependence and recognizable patterns of gut bacterial colonization in the intestinal ecosystem exist (32–35) and are known to be influenced, e.g., by dietary differences (6), only a few studies have combined the longitudinal development of representative bacterial taxa with

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parallel development of growth, body composition, and nutritional parameters in early life.

The aims of the present study were to describe patterns of microbial establishment during the first 3 years of life and to identify putative correlations of such patterns with dietary habits and physiological parameters, particularly focusing on development of body weight. We used the previously published quantitative PCR (qPCR)-based *gut low-density array* (GULDA) (36) to determine relative abundances and interbacterial relations of 31 different bacterial 16S rRNA gene targets, representing different phylogenetic levels, in fecal samples from a longitudinal cohort study of approximately 300 healthy Danish infants sampled at 9, 18, and 36 months after birth. Results were correlated with measures of growth, body composition, and nutritional records obtained for the same children, in order to reveal possible associations with the microbial composition and temporal development. Additionally, we performed the first comprehensive analysis of establishment of gut enterotypes (26, 27) in early life, using the *Prevotella/Bacteroides* ratio as a proxy for the enterotypes driven by the abundance of these genera.

MATERIALS AND METHODS

SKOT cohort. The present study is based on data and samples collected during an observational cohort study of approximately 300 apparently healthy Danish singleton term infants. The cohort, titled SKOT, based on a Danish abbreviation, was followed for a period of 3 years with visits at 9, 18, and 36 months of age, and several papers based on the cohort have been published (37–42). The study protocol was approved by the Committees on Biomedical Research Ethics for the Capital Region of Denmark (H-KF-2007-0003). Fecal samples and information on birth mode and gender, measurements of growth and body composition, food questionnaires, and background interviews were collected at the visits during the study. The numbers of participants completing the 9-, 18-, and 36-month visits were 311, 290, and 264, respectively.

DNA extraction and qPCR analysis. Total community DNA was extracted from a total of 698 fecal samples on the Maxwell 16 system using the Maxwell 16 DNA Tissue DNA purification kit (Promega Biotech AB, Sweden). The DNA concentrations were determined fluorometrically (Qubit dsDNA HS assay; Invitrogen) and adjusted to 1 ng/μl prior to use as the template in qPCR. The qPCR analysis was performed using the GULDA platform previously described (36). Briefly, each 384-well PCR plate accommodated simultaneous analysis of four DNA samples to determine the relative abundance of 31 bacterial selected 16S rRNA gene targets (Table 1) representing different phylogenetic levels. A universal bacterial primer set was included as the reference gene. All qPCRs were performed in duplicate in transparent 384-well MicroAmp optical reaction plates (Applied Biosystems) sealed with MicroAmp optical adhesive film on an ABI Prism 7900HT system (Applied Biosystems, Naerum, Denmark). Following the thermocycling program, the raw fluorescence data recorded by the SDS software were exported to the LinRegPCR program (43, 44). This software was used to perform baseline correction and calculate the mean PCR efficiency per amplicon group. This was used to calculate the initial quantities N_0 (arbitrary fluorescence units) for each amplicon using the formula $N_0 = \text{threshold}/(\text{Eff}^{CT}{}_{\text{mean}})$, where Eff_{mean} denotes the mean PCR efficiency per amplicon, threshold is the optimal "cutoff" in the exponential region, and C_T is the cycle number, where each sample exceeds this threshold. The relative abundances of the 31 specific amplicon groups were obtained by normalization to the N_0 value obtained for the universal bacterial amplicon group determined in the same array (see Fig. S1 in the supplemental material).

A universal limit of detection (LOD_U) of 10^{-5} (N_0, specific/N_0, universal) was applied to the normalized N_0 values due to qPCR analysis limitations. LOD_U was set to this value based on previous results using the GULDA setup and roughly corresponds to target C_T values above 30 cycles. All normalized N_0 values equal to or above LOD_U were included in the analysis, while samples below LOD_U were set to 0.5 LOD_U. Results were calculated as the arithmetic mean of normalized N_0 values of the two technical repeats. Differences between C_T values of technical replicates were typically less than 0.5. For samples where qPCR was successful for only one of the replicates, this value was used. Water as the template was used as the negative control. For 40 samples, no PCR amplification was detectable using the universal bacterial primer, and thus, the final number of fecal DNA samples for gut microbiota analysis was 658 representing 218, 232, and 208 at 9, 18, and 36 months, respectively. For 132 subjects, samples from all three time points were obtained. Note that a small subset of the data (obtained from 6 infants at two time points) has previously been published in order to illustrate the applicability of the PCR-based GULDA platform (36).

TABLE 1 16S rRNA gene targets included on gut low-density array (GULDA)[a]

Amplicon ID	Phylum	Class/family/genus	Species/group/family[b]
U1	Universal (all phyla)	Universal	Universal
F1b	*Firmicutes*	All	All
F2	*Firmicutes*	*Lactobacillus*	spp.
F3	*Firmicutes*	*Lactobacillus*	*L. plantarum*
F4	*Firmicutes*	*Lactobacillus*	*L. acidophilus*
F5	*Firmicutes*	*Clostridium*	*C. butyricum*
F6	*Firmicutes*	*Clostridia*	Cluster IV (*C. leptum* group)
F7	*Firmicutes*	*Clostridia*	Cluster XIVa (*C. coccoides-Eubacterium rectale* group)
F8	*Firmicutes*	*Eubacterium*	*E. hallii*
F9	*Firmicutes*	*Roseburia*	spp.
F10	*Firmicutes*	*Enterococcus*	spp.
B1	*Bacteroidetes*	All	All
B2	*Bacteroidetes*	*Bacteroides-Prevotella*	spp.
B3	*Bacteroidetes*	*Bacteroides*	spp.
B4	*Bacteroidetes*	*Bacteroides*	*B. fragilis* group
B5	*Bacteroidetes*	*Bacteroides*	*B. vulgatus*
B6	*Bacteroidetes*	*Bacteroides*	*B. thetaiotaomicron*
B7	*Bacteroidetes*	*Bacteroides*	*B. eggerthii*
B8	*Bacteroidetes*	*Bacteroides*	*B. distasonis*
B9	*Bacteroidetes*	*Prevotella*	spp.
B10	*Bacteroidetes*	*Alistipes*	spp.
A1b	*Actinobacteria*	*Bifidobacterium*	spp.
A2	*Actinobacteria*	*Bifidobacterium*	*B. bifidum*
A3	*Actinobacteria*	*Bifidobacterium*	*B. adolescentis*
A4	*Actinobacteria*	*Bifidobacterium*	*B. catenulatum/pseudocatenulatum*
A5	*Actinobacteria*	*Bifidobacterium*	*B. longum*
A6	*Actinobacteria*	*Bifidobacterium*	*B. breve*
P1	*Proteobacteria*	*Enterobacteriaceae*	Family
P2	*Proteobacteria*	*Escherichia*	*E. coli*
P3	*Proteobacteria*	*Desulfovibrio*	spp.
V1	*Verrucomicrobia*	*Akkermansia*	*A. muciniphila*
E1	*Euryarchaeota*	*Methanobrevibacter*	*M. smithii*

[a] Adapted from reference 36.
[b] spp., multiple species.

Parameters of nutrition, growth, and body composition. Information on duration of breastfeeding and level of iron supplementation was obtained from background interviews (see Table S1 in the supplemental material). Parameters of infant diet (see Table S2) were estimated from parent-completed precoded dietary records over seven consecutive days

TABLE 2 Spearman correlation analysis of the relative differences occurring in BMI from 9 to 18 months, 18 to 36 months, and 9 to 36 months (ΔBMI) with corresponding bacterial fold changes in the same period[a]

Taxon	*n*	9–18 mo		18–36 mo		9–36 mo	
		P	*R*	*P*	*R*	*P*	*R*
Firmicutes (F1b)	132	0.02*	0.20	NS		NS	
C. leptum group (F6)	132	0.02*	0.21	NS		NS	
E. hallii (F8)	131	0.03*	0.19	NS		NS	
Enterobacteriaceae (P1)	57	0.16	−0.19	0.03*	−0.28	NS	
M. smithii (E1)	25	0.04*	−0.42	NS		NS	

[a] Since fold change and Δ calculations require valid measurements for each included individual at all three time points, the number of individuals (*n*) was different for each bacterial target. *R* designates the Spearman correlation coefficient. Only bacterial taxa with *P* values below 0.05 (*) are included in the table. NS, not significant ($P > 0.05$).

(45). Dietary intake was calculated with GIES software (version 1.000d; The National Food Institute, DTU Food, Soborg, Denmark). Based on anthropometric measures described previously (39, 42), age- and gender-specific Z scores at birth and 9, 18, and 36 months (see Table S3) were calculated with the WHO Anthro software (Department of Nutrition, World Health Organization, Geneva, Switzerland). At 3 years, body composition was estimated by both dual-energy X-ray absorptiometry (DXA) and bioelectrical impedance analysis (BIA). BIA is a simple method for measuring body composition (46), and whole-body resistance, reactance, and impedance were measured using a single-frequency (50-kHz) tetrapolar BIA (Quantum III; RJL Systems, Michigan, USA) between right hand and right foot. Whole-body DXA scans were performed in a subgroup of the SKOT children ($n = 101$) with a Lunar Prodigy Advance densitometer (GE Healthcare, Madison, WI, USA) using the software enCore, version 12.30 (procedure described in detail by Jensen et al. [39]). Here, DXA fat-free mass (FFM) and fat mass (FM), resistance index (height^2/resistance), and FFM and FM predicted from BIA were all used as measures for body composition at 3 years.

Assessment of changes in the gut microbial composition. The normalized N_0 values obtained for each bacterial taxon were $\log_{10}$ transformed and used as input for multivariate principal component analysis (PCA) using LATENTIX version 2.11 (Latent5 Aps, Frederiksberg, Denmark). Univariate statistical analysis was performed using the GraphPad Prism software (version 5.03; GraphPad Software Inc., La Jolla, CA). Only individuals for whom samples at all three examinations were available were included. Specific primer results, which never exceeded LOD_U at either 9, 18, or 36 months for a given individual, were excluded from further analysis. Consequently, the number of individuals for analysis of each bacterial taxon ranged from $n = 25$ to $n = 132$. Fold changes (FC) for specific gene targets were calculated as the pairwise ($\log_2$) ratio of normalized, but not $\log_{10}$-transformed, abundances at 9, 18, and 36 months, giving three ratios: 18 months/9 months, 36 months/9 months, and 36 months/18 months. Mean and corresponding standard error of the mean (SEM) values were calculated, and a one-sample *t* test was performed to test if the fold changes differed significantly from zero. The Wilcoxon signed-rank-sum test was performed as an alternative when data were not normally distributed. Correction for multiple testing (47) was performed for the 90 comparisons (3×30) presented in Fig. 2. Three statistical significance levels were employed: $P < 0.05$, $P < 0.01$, and $P < 0.001$. Spearman correlations *R* and corresponding *P* values between bacterial fold changes from 9 to 18 months and from 18 to 36 months, respectively, and changes in parameters of growth and body composition and nutritional parameters (see Tables S1, S2, and S3 in the supplemental material) were calculated using GraphPad (Table 2). The Mann-Whitney test was used to compare effects of continued or terminated breastfeeding at the 9-month examination on the relative abundances of all bacteria at 9, 18, and 36 months, respectively.

Spearman correlations at 9, 18, and 36 months. For the time-independent analysis, all valid samples for each of the three time points, 9, 18, and 36 months, were included ($n > 200$ in each age group). Pairwise correlations between all measured SKOT parameters, including the microbiota, at all three time points were performed using GraphPad. Spearman correlations were applied for all pairwise correlations. False discovery rates (FDR) were calculated by a classical one-stage method (47).

The ratios between relative abundances of the *Prevotella* (B9) and *Bacteroides* (B3) targets were calculated as a proxy for the corresponding *Prevotella*- or *Bacteroides*-driven gut enterotypes (26). The logged relative abundances of *Bacteroides* spp. and *Prevotella* spp., frequency distributions of *Bacteroides* spp. and *Prevotella* spp., and corresponding ratio (P/B) were calculated for $n = 69$, 84, and 130 individuals, at 9, 18, and 36 months, respectively. A Kernel density plot was fitted to all histograms using the Kernel add-in package for Microsoft Excel. Characterization of the frequency distributions as uni- or bimodal was tested with a dip test, calculated by the R package diptest (R package version 0.75-4, based on Fortran and S-plus from Dario Ringach, New York University).

Samples from the $n = 79$ individuals giving qPCR results for both B3 (*Bacteroides* spp.) and B9 (*Prevotella* spp.) at both 18 and 36 months were stratified as either high- or low-P/B enterotypes and investigated for their putative cooccurrence with specifically high or low levels of physiological parameters (nutrition, growth, or body composition). Finally, the fold change from 18 to 36 months using the same stratification was correlated with the longitudinal development of all parameters of nutrition, growth, and body composition from 18 to 36 months.

RESULTS AND DISCUSSION

Development of the gut microbiota. Although a quite extensive amount of literature on the possible factors involved in microbiota development in early life exists (for reviews, see references 48 and 49), these studies typically focus on microbial colonization immediately after birth (50, 51), during weaning at 4 to 6 months (3, 52), or up to 1 year (7) and, in a single recent study, 2 years of age (53). To our knowledge, no previous studies including numbers of participants as high as those in the present study have focused on the development occurring in the microbiota between infancy and 3 years of age.

We observed a clear change in the microbiota during this period, in particular from age 9 to 18 months (Fig. 1). The 9-month samples appeared to cluster less closely together than the later samples and were characterized by more lactic acid bacteria and enterobacteria than seen for samples taken at ages 18 and 36 months. The two other age groups comprised a higher number of different microbes, including both *Firmicutes* and *Bacteroidetes*. In line with this, the majority of specific changes in abundances of given bacterial taxa occurred between 9 and 18 months (Fig. 2; see also Fig. S2 in the supplemental material). We observed a consistent and significant increase of several species within the *Bacteroidetes* phylum, which is consistent with reported findings seen after introduction of complementary feeding (6, 54). Additionally, we observed a significant decrease in the relative abundance of *Bifidobacterium* spp. (FC, −2.56; $P < 0.001$). Within these, *Bifidobacterium longum* (FC, −2.77; $P < 0.001$) and *Bifidobacterium breve* (FC, −6.47; $P < 0.001$) were observed to decrease, while *Bifidobacterium adolescentis* (FC, 2.33; $P < 0.01$) and *Bifidobacterium catenulatum* (FC, 4.00; $P < 0.001$) increased during the period from 9 to 36 months of age, indicating that during early childhood, the conditions in the gut and/or the diet changes in ways that favor the latter species of bifidobacteria later in life. For example, breast milk is known to contain bifidogenic human milk oligosaccharides (HMOs), which are atypical carbohydrates, re-

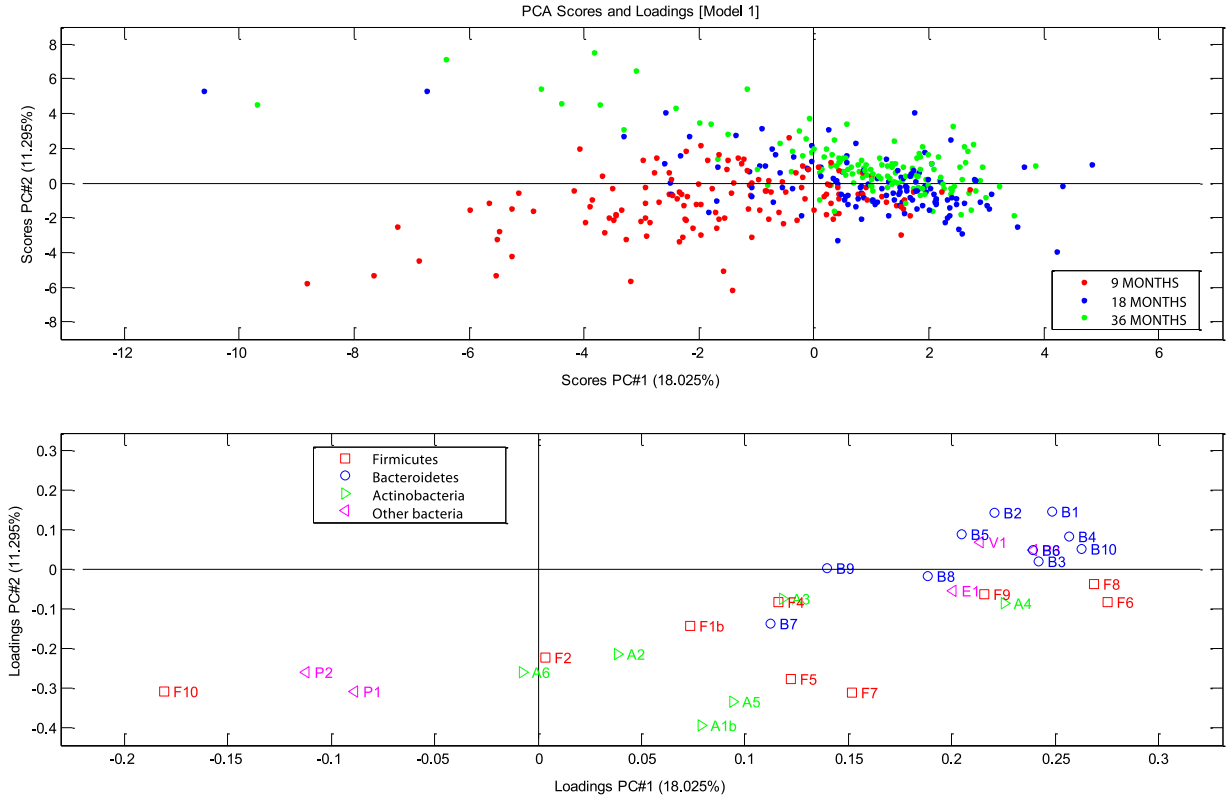

FIG 1 Principal component analysis (PCA) of the GULDA microbiota. Upper plot: scores (individuals); lower plot: loadings (bacterial 16S rRNA gene targets). This figure shows the two primary principal components, PC1 and PC2, which explain 18.02% and 11.29% of data variation, respectively. Bacterial targets primarily associated with the lower left quadrant and thus relatively highly abundant in the 9-month samples were *Enterococcus* spp. (F10), *Enterobacteriaceae* (P1), and *Escherichia coli* (P2) and, to a lesser extent, *B. breve* (A6) and *Lactobacillus* spp. (F2). Bacterial targets appearing in higher abundances in the 36-month samples were the *Bacteroidetes* (B1), the *Bacteroides-Prevotella* group (B2), *Bacteroides* spp. (B3), *B. fragilis* group (B4), *B. vulgatus* (B5), *Bacteroides thetaiotaomicron* (B6), *Alistipes* spp. (B10), *A. muciniphila* (V1), and *Desulfovibrio* spp. (P3). Complete explanations for all labels are given in Table 1.

sistant to enzymatic hydrolysis in the upper gastrointestinal tract (55, 56). *B. longum*, *Bifidobacterium bifidum*, and *B. breve* are particularly abundant in breastfed children (57) and known to be highly proficient in capturing and utilizing HMOs as their sole carbon source, while *B. adolescentis* is unable to degrade these oligosaccharides (58).

Lactobacillus spp. (FC, −1.33; $P < 0.05$) and *Enterobacteriaceae* (FC, −4.21; $P < 0.001$) were found to decrease between 9 and 18 months (Fig. 2), while an increase was observed for the butyrate-producing taxa *Clostridium leptum* group (FC, 2.32; $P < 0.001$), *E. hallii* (FC, 3.65; $P < 0.001$), and *Roseburia* spp. (FC, 4.62; $P < 0.001$) from age 9 to 36 months. This is in accordance with previous findings seen at cessation of breastfeeding and introduction of formula feeding and/or cow's milk (1, 2, 5, 24, 54, 59–61). Conversely, the butyrate-producing *Clostridium coccoides* group (FC, −3.14; $P < 0.001$) was seen to be reduced between 9 and 18 months. A previous report (62), based on a cross-sectional study of 40 children, indicates that the *C. coccoides* group increases until 6 months of age and thereafter remains at a stable level.

The fact that we observed significant changes still occurring from 18 to 36 months (Fig. 1 and 2) suggests that convergence toward adult-like stability, characterized by high levels of *Firmicutes* and *Bacteroidetes* and smaller fractions of *Actinobacteria*, *Proteobacteria*, and *Verrucomicrobia* (63–65), was still occurring during this period. This is in line with a recent large cross-sectional study of humans from different age groups showing that bacterial communities of the gut evolve toward adult-like configurations during the first 3 years of life (66) but contradicts older reports proposing that full stability is reached already at 12 months (6, 7, 67).

Correlations between relative abundance of bacterial groups and physiological parameters. In agreement with previous studies from other researchers (58), continued breastfeeding at 9 months was associated positively with high relative abundances of *Lactobacillus* spp., *Bifidobacterium* spp., and *B. longum* at 9 months (Fig. 3). Additionally, compared to infants no longer breastfed at 9 months, infants still breastfed at 9 months had lower levels of a number of butyrate-producing taxa, including *C. leptum* group, *C. coccoides* group, *E. hallii*, and *Roseburia* spp. Breastfeeding at 9 months was also associated with lower levels of *Desulfovibrio* spp. and *Akkermansia muciniphila*, as well as of the *Bacteroidetes* phylum and several taxa therein. For the *C. coccoides* group and some of the *Bacteroides* species, the differences were still present after 18 months, while at 36 months the breastfeeding history no longer influenced the microbiota (Fig. 3). Breastfeeding has been shown to significantly reduce the risk of overweight/obesity later in childhood as well as in adult life (68); however, conflicting reports of the role of breastfeeding in obesity also exist (69). We speculate that the observation that continued breastfeed-

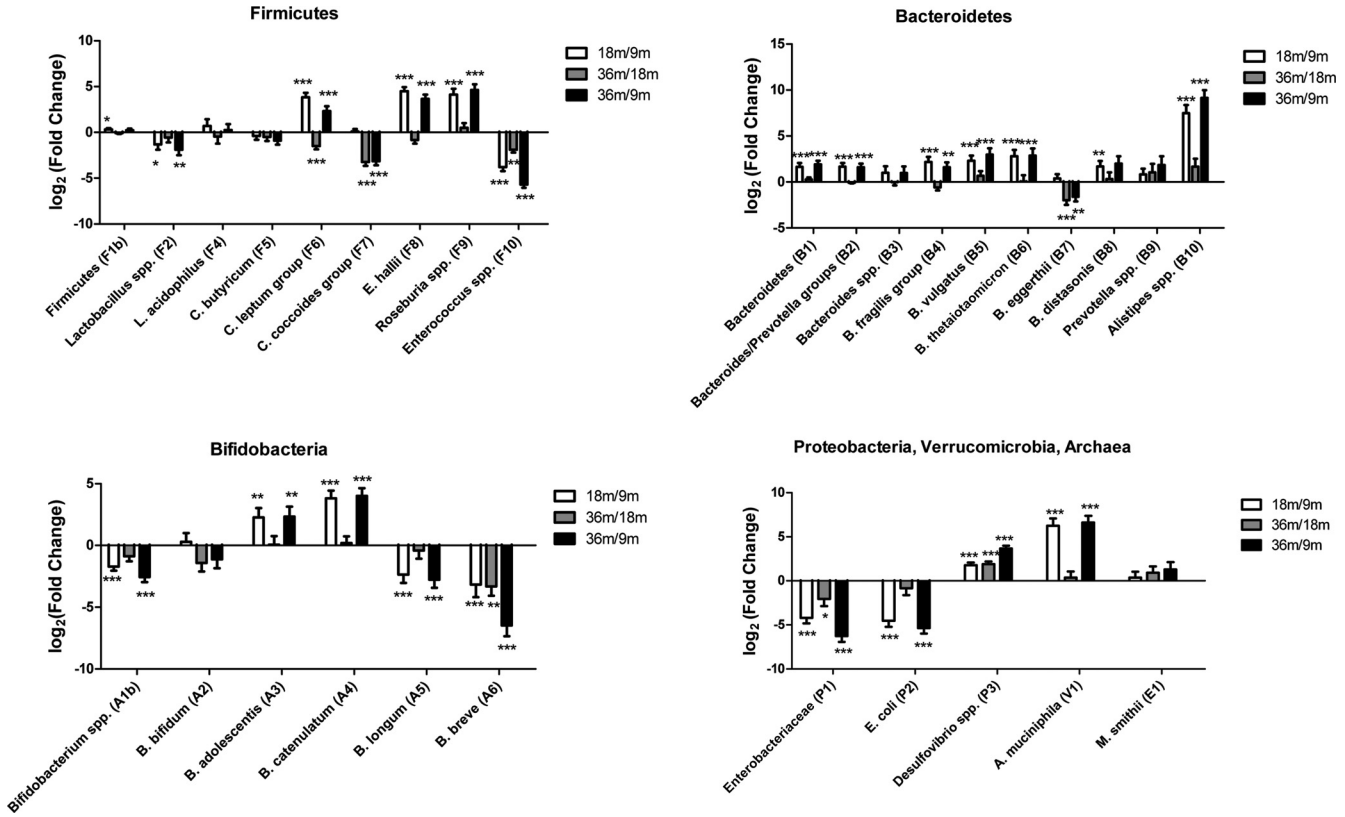

FIG 2 Progressive development of the gut microbial composition. Log_2(fold changes) of microbial 16S rRNA gene targets occurring from 9 to 18 months (white) and 18 to 36 months (gray) and cumulative values from 9 to 36 months (black). Statistical significance of one-sided *t* tests: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Data were corrected for multiple testing, using a maximal false discovery rate of 5%.

ing at 9 months delays progression of specific bacterial taxa may be of relevance for development and later-life health.

We observed only very few correlations between the abundances of specific gut bacteria and the physiological parameters measured (see Fig. S3 in the supplemental material). Significant associations with $P < 0.001$ and false discovery rates (q) below 0.08 were observed at 9 months, where duration of breastfeeding (breast milk days, indicating the number of days with either partial or exclusive breastfeeding as estimated by the mothers) was shown to correlate positively with *Lactobacillus* and *Bifidobacterium* targets. Less significantly ($P < 0.01$, $q < 0.32$), negative associations with duration of breastfeeding were seen for *C. leptum* group, *E. hallii*, *Roseburia* spp., *Bacteroides/Prevotella* groups, *Bacteroides fragilis*, *Bacteroides vulgatus*, *Desulfovibrio* spp., and *A. muciniphila*.

Supporting these observations, many of the opposite trends for correlations were observed between these bacterial targets and the intake of infant formula, including a negative correlation ($P < 0.001$, $q < 0.08$) with the abundance of *Lactobacillus* spp. Additionally, the data reflected that duration of breast milk consumption (breast milk days) was negatively associated with the overall energy intake, as previously reported (38). We have previously shown that the breastfed infants had lower body mass indexes (BMIs) at both 9 and 18 months (41). No other significant correlations were found between the gut microbiota and nutritional parameters, or measures of growth and body composition (including DXA and BIA examinations at 36 months), gender, or birth mode (vaginal versus caesarean) at any of the three time points (data not shown). Previous reports on correlations between BMI and gut microbiota composition in young children exist (70, 71); however, these were observed in cross-sectional cohort studies where sampling was focused on high versus normal BMI, while the infants included in the present study constituted a younger and leaner population, with only 8% classified as overweight (72).

Exploiting the longitudinal observations from the present study, we found significant ($P < 0.05$) positive correlations between increase of body mass index (ΔBMI) and the increase of the *Firmicutes* phylum, the *C. leptum* group, and *E. hallii* (belonging to the *C. coccoides* group) between 9 and 18 months (Table 2). Additionally, increases in *Methanobrevibacter smithii* were negatively correlated with ΔBMI from 9 to 18 months, while reductions in *Enterobacteriaceae* were associated with higher ΔBMI from 18 to 36 months. Similar results were obtained for ΔBMI-for-age Z score (ΔBAZ) and Δweight-for-length Z score (ΔWFL) but not for changes in Z scores for weight for age (WAZ), length for age (HAZ), subscapular skinfold for age (SSZ), or triceps skinfold for age (TSZ) (data not shown). No other significant correlations between changes in body compositional measures and changes in nutritional parameters and/or bacterial targets were observed. Although excessive weight gain during the first 6 months after birth has been shown to be particularly predictive of later obesity (73–76), we found no significant characteristics in the

	9 months	18 months	36 months
Firmicutes			
Firmicutes (F1b)	0,566	0,182	0,4265
Lactobacillus spp. (F2)	0,0001***	0,1736	0,4136
L. acidophilus (F4)	0,6249	0,172	0,9128
C. butyricum (F5)	0,8274	0,0561	0,6691
C. leptum group (F6)	0,0267*	0,4015	0,5951
C. coccoides group (F7)	0,0021**	0,0097**	0,8153
E. hallii (F8)	0,0193*	0,4321	0,6329
Roseburia spp. (F9)	0,0316*	0,4482	0,6901
Enterococcus spp. (F10)	0,0731	0,0379*	0,5529
Bacteroidetes			
Bacteroidetes (B1)	0,016*	0,243	0,2087
Bacteroides/Prevotella groups (B2)	0,0177*	0,0128*	0,1326
Bacteroides spp. (B3)	0,126	0,3384	0,0709
B. fragilis group (B4)	0,0004***	0,0984	0,8973
B. vulgatus (B5)	0,0345*	0,0115*	0,1967
B. thetaiotaomicron (B6)	0,0016**	0,017*	0,3561
B. eggerthii (B7)	0,1241	0,9103	0,8546
B. distasonis (B8)	0,2906	0,1402	0,8114
Prevotella spp. (B9)	0,3372	0,1112	0,076
Alistipes spp. (B10)	0,1745	0,2494	0,3157
Bifidobacteria			
Bifidobacterium spp. (A1b)	0,0002***	0,51	0,5306
B. bifidum (A2)	0,6315	0,4082	0,8208
B. adolescentis (A3)	0,8294	0,9744	0,946
B. catenulatum (A4)	0,4334	0,4076	0,7047
B. longum (A5)	0,0477*	0,8546	0,2187
B. breve (A6)	0,7623	0,467	0,6985
Other bacteria			
Enterobacteriaceae (P1)	0,6179	0,6436	0,0523
E. coli (P2)	0,6053	0,555	0,1425
Desulfovibrio spp. (P3)	0,0449*	0,1072	0,9721
A. muciniphila (V1)	0,0451*	0,0863	0,811
M.smithii (E1)	0,0753	0,3251	0,6047

Increased in breastfed at 9 months

Decreased in breastfed at 9 months

*p<0.05; **p<0.01**; ***p<0.001

FIG 3 Effect of breastfeeding on infant gut microbiota. *P* values of Mann-Whitney statistical tests addressing differences between relative bacterial abundances at 9, 18, and 36 months, dependent on whether or not the infants were still breastfed at the 9-month examination. Green indicates an increase in children breastfed at 9 months, and red indicates a corresponding decrease.

microbiota after 9 months, which corresponded to changes in BAZ or WAZ between birth and 9 months (data not shown).

The clostridial targets selected for this study represent colonic butyrate-producing bacteria, assisting in the conversion of polysaccharides to monosaccharides and short-chain fatty acids (SCFA) constituting energy for the host (77, 78). The development of abundances of the selected taxa *C. butyricum*, *C. leptum* group, *C. coccoides* group, *E. hallii*, and *Roseburia* spp. (the two last taxa belonging to the *C. coccoides* group) was very different for the different targets (Fig. 2). Significant differences in carbohydrate metabolism and butyrate production between different clostridia have been demonstrated to be of relevance to the pathogenesis of obesity (79). It remains to be established whether certain dietary compounds, arguably containing high concentrations of specific complex polysaccharides, are specifically subjected to catabolism by the *C. leptum* group, *E. hallii*, and *Roseburia* spp. between 9 and 18 months, as suggested by the data (Fig. 2).

Correlation between bacterial groups at 9, 18, and 36 months. Correlations between the abundances of the bacterial 16S rRNA gene targets were investigated for each of the three age groups (Fig. 4). Although there were more differences between the 9-month pattern and the two later groups, differences between 18 and 36 months were also observed.

At 9 months, we found coabundance (positive Spearman correlations) between the butyrate-producing *Firmicutes C. leptum* group, *C. coccoides* group, and *Clostridium butyricum*. Similarly, coabundance was seen for many of the *Bacteroides* species, with the notable exception of *Bacteroides eggerthii*. With this specific exception, a high abundance of *Firmicutes* was also clearly associated with a low abundance of *Bacteroides* species. While the other *Bacteroides* species were increasing in abundance during the study, *B. eggerthii* was reduced (Fig. 2). The reverse cooccurrence of *B. eggerthii* and the other *Bacteroides* species was no longer as significant at 36 months (Fig. 4), indicating that later in life, the envi-

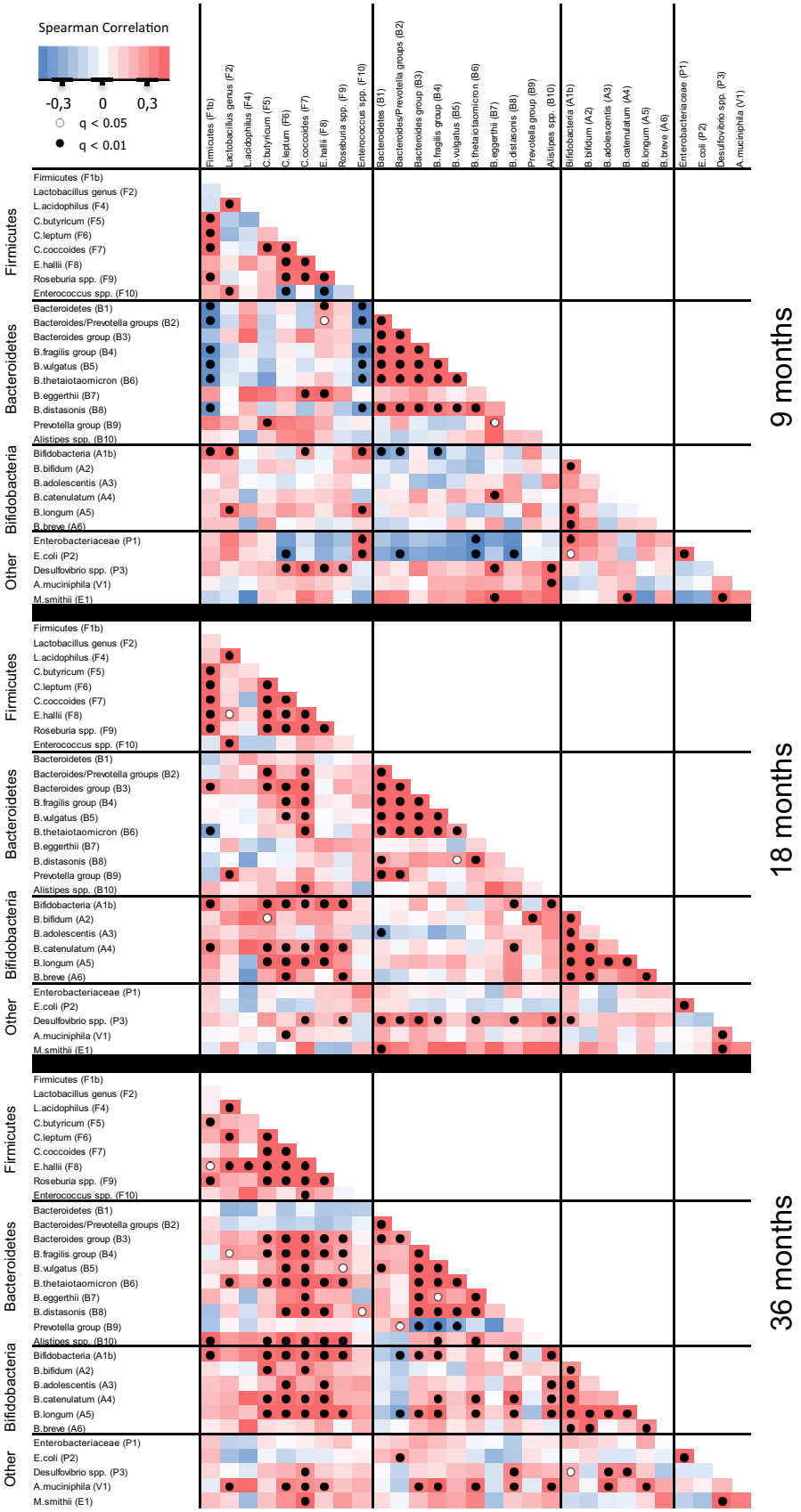

FIG 4 Spearman pairwise correlation map of measured bacterial 16S rRNA gene targets at 9, 18, and 36 months. Because each time point was analyzed separately, the included number of individuals was >200 at each of the three samplings. The color gradient denotes Spearman *R* values. Dots indicate significant correlations, corrected for false discovery rates (*q*).

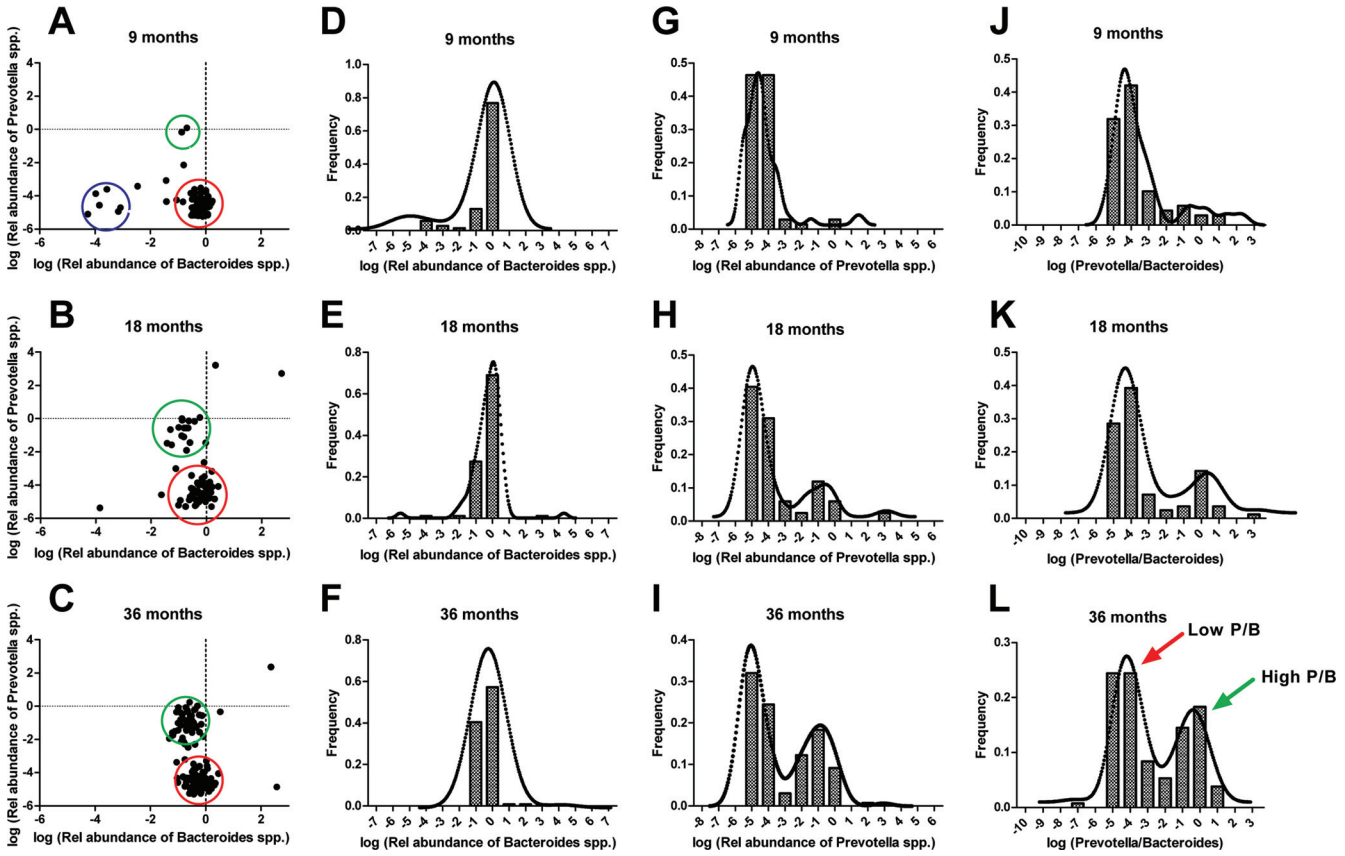

FIG 5 Enterotype defined as P/B development from 9 to 36 months. Relative abundances of log(*Bacteroides* spp.) and log(*Prevotella* spp.) show a distinct development from 9 to 36 months (A to C), moving from low relative abundances of both groups (blue circle) to a *Bacteroides*-prevalent microbiota (red circle) at 9 months. From 9 to 36 months, an increasing subgroup of *Prevotella*-prevalent samples appear (green circle), indicating segregation of specific individuals from a *Bacteroides*-driven into a *Prevotella*-driven enterotype. This progressive development is also evident from the corresponding histograms of frequency distributions of log(*Bacteroides* spp.) (D to F) and log(*Prevotella* spp.) (G to I) abundance, and particularly from the distributions of the logged P/B (J to L). The dotted curve in panels D to L shows a Kernel density plot, which is a modification of the histogram patterns, supporting the underlying statistical distributions found. Panels A, D, G, and J (9 months) represent 69 individuals; panels B, E, H, and K (18 months) represent 84 individuals; and panels C, F, I, and L (36 months) represent 130 individuals, as only individuals with relative abundances of *Bacteroide*s spp. and *Prevotella* spp. exceeding the detection limit were included.

ronment in the gut no longer represses this organism more than the other *Bacteroides* organisms. Additionally, the clear association between a high abundance of *Firmicutes* and enterococci and a low abundance of *Bacteroides* spp. seen at 9 months has disappeared at 18 and 36 months, indicating that it is particularly in infancy that these groups are mutually exclusive of each other.

Bacteroides spp. and *Clostridiales* spp. have previously been seen to cocluster in the so-called enterotype driven by the abundance of *Bacteroides* spp. in healthy human adults (26). This is in agreement with our data from age 18 months and becomes even clearer at 36 months; however, at 9 months we found no coabundance of these groups (Fig. 4). It seems plausible that the development of this coabundance is a logical consequence of adaptation to a Western-type diet after weaning. However, analysis of possible correlations between bacterial targets and the investigated nutritional parameters (see Table S2 in the supplemental material) did not result in statistically significant cooccurrences (data not shown).

We found it noteworthy that at 9 months, when *Bifidobacterium* spp. in general were most abundant (Fig. 2), there was a clear cooccurrence of particularly *B. longum* with the other lactic acid-producing taxa *Lactobacillus* spp. and *Enterococcus* spp.; however, no cooccurrence between specific species of *Bifidobacterium* was seen (Fig. 4). This pattern was reversed at 18 and 36 months, when the cooccurrence with lactobacilli and enterococci was no longer present, while cooccurrence of the specific *Bifidobacterium* species *B. longum* with *B. bifidum*, *B. adolescentis*, and *B. catenulatum*, and further of *B. bifidum* with *B. catenulatum* and *B. breve* was evident. Interestingly, these cooccurrences existed independently of the fact that the average abundance of certain *Bifidobacterium* species was increased during the experimental period, while others were reduced as discussed above (Fig. 2).

Enterotype development in the infant gut. Lately, the existence of three distinct enterotypes, driven by the abundance of *Bacteroides* spp., *Prevotella* spp., and *Ruminococcus* spp., respectively, has been given particular attention (26). While evidence is mounting in support of the distinction between the *Bacteroides*- and *Prevotella*-driven groupings, the existence of the third group, driven by the abundance of *Ruminococcus* spp., is not as clearly supported (80). In the present study, we used the relative abundance between *Prevotella* spp. and *Bacteroides* spp., measured as the *Prevotella/Bacteroides* ratio (P/B), as a proxy for the enterotypes driven by these two genera as proposed by Arumugam et al. (26). It is important to note that we do not mean to propose that

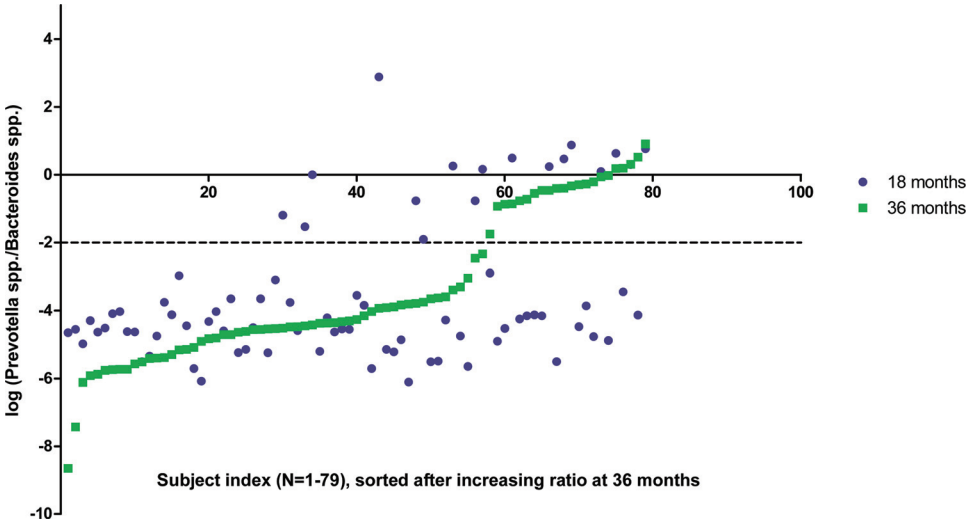

FIG 6 Changes in P/B occurring between age 18 and 36 months The 79 individuals giving qPCR results for both 18 (blue) and 36 (green) months were sorted after increasing logged P/B at 36 months. Samples above the dotted line belong to the high-P/B group (*Prevotella*-driven enterotype), while samples below this line belong to the low-P/B group (*Bacteroides*-driven enterotype). Forty-eight of 79 individuals remained in the low-P/B group, while 8/79 individuals remained in the high-P/B group from age 18 to age 36 months. Fourteen of 79 and 9/79 individuals shifted from low to high P/B, or from high to low P/B, respectively.

the enterotypes are characterized solely by the abundance of these taxa but merely that their abundance can be used as a marker for more complex differences characterizing these two types of intestinal bacterial communities. For the first time, this approach allowed addressing the establishment of enterotypes during infancy.

In agreement with the existence of enterotypes, we observed a negative correlation between *Prevotella* spp. and *Bacteroides* spp. at 36 months of age but not at 9 or 18 months (Fig. 4). The relative abundances of both *Prevotella* spp. and *Bacteroides* spp. are below detection limits at birth, given the absence of these bacteria in the prenatal environment of the maternal uterus (48). We propose that *Bacteroides* spp. colonize better than *Prevotella* spp. between birth and 9 months (9), as almost all individuals were characterized by a low P/B at 9 months of age (Fig. 5A). At 18 months, a smaller subset of individuals established a higher P/B (Fig. 5B), a pattern which was even more pronounced after 36 months, where two distinct groups appeared (Fig. 5C). We observed a unimodal distribution of *Bacteroides* abundances at all three sampling points (Fig. 5D to F), while an increasingly bimodal pattern of *Prevotella* abundances (Fig. 5G to I) developed from 9 to 36 months. The ratio of logged P/B values similarly showed an increasingly bimodal pattern with age, with high-P/B samples characterized by a logged ratio above a level of -2 (P/B $>$ 0.01) and low-P/B samples below -2 (P/B $<$ 0.01) (Fig. 5J and K). When tested statistically for the presence of bimodal distribution, there was significance at the 36-month *Prevotella* abundance ($P = 0.004$) (Fig. 5I) and an even clearer bimodality for the 36-month P/B ($P = 6.6E^{-5}$) (Fig. 5L), but not at the earlier time points. Consequently, development of the two enterotypes starts between 18 and 36 months and is driven by changes in *Prevotella* spp., rather than *Bacteroides* spp. However, stratification of samples into P/B types at either 18 or 36 months did not reveal any additional correlations between bacterial 16S rRNA gene targets and host physiological phenotypes.

Out of the 79 individuals for whom the P/B could be calculated for both 18 and 36 months, 70% remained in the same P/B group between 18 and 36 months, while 18% and 11%, respectively, shifted their enterotypes from low P/B to high P/B or vice versa (Fig. 6). Previous studies have shown that although the adult gut microbiota of any individual is quite resilient to major perturbations, enterotypes may shift in a few individuals when measured over longer periods of time (81, 82) and by long-term dietary intervention schemes (80). In the present study, we did not identify any correlation of enterotype with diet or BMI. No data on antibiotic treatment were collected for the cohort; however, since antibiotic treatment is known to induce major changes in the composition of the infant gut microbiota (60, 83, 84), it cannot be excluded that antibiotic usage prior to 18- or 36-month samplings influenced the observed shifts. Nevertheless, our results (Fig. 6) strongly support the notion that between 18 and 36 months, the P/B enterotype is still not as stably established as reported in adults (28). In light of recent findings showing that enterotypes driven by *Bacteroides* spp. and *Prevotella* spp. affect risk markers for atherosclerosis (85) and that gut microbiome composition correlates with obesity and metabolic markers in adults (86), we propose that the establishment of microbiota during infancy may affect health status in adult life.

Concluding remarks. We have studied the establishment of intestinal microbiota in a large cohort of Danish infants and analyzed the microbial data in relation to a vast amount of dietary and physiological measures. We demonstrate significant differences in microbiota composition between infants either breastfed or no longer breastfed at 9 months but additionally show that the effects of breastfeeding on the microbiota are no longer prevalent at age 36 months. Positive correlations between increases in BMI, *C. leptum* group, and *E. hallii* were observed from 9 to 18 months, indicating that these butyrate-producing groups may contribute importantly to host energy harvest. Additionally, we show for the first time that human enterotypes, expressed as a bimodal distribution of the *Prevotella/Bacteroides* ratio, start being established between 18 and 36 months of age. In this period, where we observe an ongoing development of the microbiota toward an adult-like

composition, enterotypes are still more susceptible to shifting than previously seen for adults.

Considering the increasing evidence supporting a key role of gut microbiota composition in human health, the presented data constitute an important new body of knowledge on microbiota development during infancy, which is likely to constitute a window where the microbiota can be more significantly influenced by intervention. In this context, the current development in next-generation sequencing is expected to contribute importantly to our understanding of human microbiome establishment during the coming years.

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