Three Main Factors Define Changes in Fecal Microbiota Associated With Feeding Modality in Infants

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ABSTRACT

Objectives: There are many differences in the fecal infant microbiota associated with various feeding methods. The aim of this study was to examine the major differences in the fecal microbiota of breast-fed (BF) and formula-fed (FF) infants and to describe the principal bacterial components that would explain the variability in the predominant bacterial families and genus clusters.

Methods: Fecal samples from 58 infants, 31 of whom were exclusively BF and 27 of whom were exclusively FF with a standard formula in agreement with the European Society for Pediatric Gastroenterology, Hepatology, and Nutrition recommendations, were analyzed by fluorescent in situ hybridization combined with flow cytometry. Principal component analysis was used to maximize the information gained for the predominant bacterial families and genus clusters using a minimal number of bacterial groups.

Results: The predominant detected group was *Bifidobacterium*, followed by Enterobacteriaceae and *Bacteroides* in both BF and FF infants. The *Lactobacillus* group was the only independent variable associated with FF infants. We also found that 3 principal components were sufficient to describe the association between the bacterial group, genus, and species studied in BF and FF infants; however, these components differed between BF and FF infants. For the former, the 3 factors found were *Bifidobacterium*/Enterobacteriaceae, *Lactobacillus*/*Bacteroides*, and *Clostridium coccoides*/*Atopobium*; for the latter, *Bifidobacterium*/Enterobacteriaceae, *Bacteroides* and *C coccoides* were observed.

Conclusions: There is a clear clustering of components of infant microbiota based on the feeding method.

Key Words: feeding methods, fluorescent in situ hybridization, infant fecal microbiota

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Microbiota plays an important role in human health and nutrition by preventing colonization of the gut by potential pathogenic microorganisms and preserving the health of the host through interactions with the immune system (1,2). The development of microbiota occurs primarily during infancy, and microbial dysbiosis has been linked to several disorders such as inflammatory bowel disease (3,4), irritable bowel syndrome (5), stomach cancer (6), mucosa-associated lymphoid tissue lymphoma (7), obesity (8,9), and necrotizing enterocolitis (10).

Bacterial colonization of the gastrointestinal tract is influenced by many factors such as infant diet (breast milk vs infant formula), mode of birth, perinatal antibiotics, mucosal maturation, age, country of birth, and geographical origin (1,11). Given the importance of the intestinal microbiota and its effect on infant health status, there is a considerable interest in determining the microbiota composition. It is well known that breast-fed (BF) infants differ widely in their microbiota compared to formula-fed (FF) infants (12,13). FF infants develop a complex fecal microbiota with higher levels of the facultative anaerobes *Bacteroides* and *Clostridium* than BF infants. The predominance of *Bifidobacterium* is also common in FF infants, although in lower number and frequency than BF infants of the same age (12,13); however, recent metagenomics studies have raised questions about the actual levels of *Bifidobacterium* (14,15).

The aim of this study was to determine the fecal microbiota in both BF and FF infants using fluorescent in situ hybridization combined with flow cytometry (FISH-FC), which allows the precise evaluation of living bacteria. In addition, the present work was undertaken to show that the main components of fecal microbiota explain the variability in BF and FF infants as a result of variables that include different bacterial groups, genus, and species as detected by FISH-FC.

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TABLE 1. Baseline demographic data of infants involved in the study

	Sex		Gestation age, wk		Birth weight, kg	
	Boys	Girls	Mean	SD	Mean	SD
Breast-fed infants, n = 31	17	11	39.3	1.2	3.3	0.4
Formula-fed infants, n = 27	10	17	39.2	1.4	3.1	0.4

SD = Standard deviation.

METHODS

Subjects

Fifty-eight healthy infants born at the Neonatology Unit of University Hospital Virgen de la Arrixaca (Murcia, Spain) were selected: 31 were exclusively BF and 27 were exclusively FF. Based on the variance of the main outcome variable, that is, *Bifidobacterium* percentage in feces as determined by FISH-FC for Spanish infants (1), and assuming a type 1 error of $\alpha = 0.05$ and a power of 80% ($\beta = 0.2$), the minimum number of subjects per group was 30. Initially, we recruited 32 BF infants and 28 FF infants, but the number of subjects decreased to 31 and 27, respectively, because of fecal samples that were not fixed properly. The infant formulas used were in accordance with the recommendations of the European Society for Pediatric Gastroenterology, Hepatology, and Nutrition. The inclusion criteria were as follows: birth at full term (40 ± 2 weeks' gestation), normal birth weight (≥ 2.5 kg), and vaginal delivery. None of the infants received antibiotics during the study period. Table 1 shows the demographic characteristics of the infants involved in the study.

This study was conducted according to the guidelines of the Declaration of Helsinki, and all procedures involving human subjects were approved by the ethical committees on clinical research from Hospital Virgen de la Arrixaca. All parents gave their written informed consent to participate in the study.

Sample Collection

Fecal samples were collected from each infant at 12 weeks of age. Freshly soiled diapers were placed under anaerobic conditions at 4°C for a maximum of 4 hours before processing for cell fixation (2). Aliquots of 1 g of feces samples were added to 9 mL of phosphate-buffered saline (PBS). The suspension was mixed to complete homogeneity, and 0.2 mL of the suspension was added to 0.6 mL of 4% paraformaldehyde in PBS. After an overnight incubation at 4°C, the suspensions fixed in paraformaldehyde were stored at −80°C (1).

FISH-FC Analysis

Fecal bacteria populations were assessed by FISH-FC analysis as described by Fallani et al (1). In brief, 400 μL of the fixed suspension was mixed with 600 μL of PBS. Before hybridization, the cells were pelleted and resuspended in a volume of 1 mL. After washing in Tris-ethylenediaminetetraacetic acid buffer, the pellets were resuspended in Tris-ethylenediaminetetraacetic acid buffer containing 1 mg/mL of lysozyme and incubated for 10 minutes at room temperature. The cells were then washed in PBS and equilibrated in the hybridization solution. A 50-μL aliquot of this suspension was used for FISH-FC with control- and group-specific probes (ThermoFisher, Hesse, Germany). The EUB 338 probe was used as a positive control for hybridization, and the NON 338 probe was used as a negative control (1,16,17). These oligonucleotide probes were covalently linked at their 5′ end with either 6-FAM or Cy5 (ThermoFisher). Similar to Fallani et al (1), a panel of 10 group- and species-specific probes covalently linked with Cy5 at their 5′ end was used to assess the microbiota composition (Table 2) (18–25).

Hybridization was performed in a 96-well microtiter plate overnight at 35°C in the hybridization solution containing 4 ng/μL of the appropriate probes. Following hybridization, 150 μL of hybridization solution was added to each well, and cells were pelleted and washed to remove any nonspecific binding of the probe by incubating the bacterial cells at 37°C for 20 minutes in the washing solution. Finally, the cells were pelleted and resuspended in PBS. The samples were analyzed in a FACSCanto II flow cytometer (Becton Dickinson, Franklin Lakes, NJ) through the instrumental scientific service of the University of Granada as described by Fallani et al (1). Enumeration of different bacterial families, genus, clusters, or species was performed by FISH-FC

TABLE 2. Panel of probes used for in situ determination of infant gut microbiota

Probes	Sequence from 5′ to 3′ end	Targeted groups	Ref
Bif164	CATCCGGCATTACCACCC	*Bifidobacterium* genus	(18)
Bac303	CCAATGTGGGGACCTT	*Bacteroides* group	(19)
Enter1432	CTTTTGCAACCCACT	Enterobacteriaceae	(20)
Str493	GTTAGCCGTCCCTTTCTGG	*Streptococcus* group	(21)
Lab158	GGTATTAGCAYCTGTTTCCA	*Lactobacillus group*	(22)
Ato291	GGTCGGTCTCTCAACCC	*Atopobium* cluster	(23)
Erec482	GCTTCTTAGTCARGTACCG	*Clostridium coccoides* group	(21)
Clep866	GGTGGATWACTTATTGTG	*Clostridium leptum* group	(24)
Cpef191	GCTCCTTTGGTTGAATGATG	*Clostridium perfringens*	(25)
Cdif198	TCCATCCTGTACTGGCTCACC	*Clostridium difficile*	(25)

combining a specific probe labeled with Cy5 together with the EUB 338 6-FAM probes in the same tube.

Statistical Analysis

Data are expressed as the mean and standard error of the mean for the proportions of cells that were hybridized with each of the 10 oligonucleotide probes relative to the total bacteria (1). Paired *t* tests were used to compare any differences in variables (bacterial groups) detected in the feces of BF or FF infants.

Principal component analysis was used to maximize the information gained for the predominant bacterial families and gene clusters using a minimal number of bacterial groups. This mathematical model calculates new variables (principal components) that account for the variability in the data and enables the study of covariances or correlations between variables (bacterial groups). The combination of bacterial groups with the greatest amount of variability is the first principal component. The subsequent components (second and third principal components) describe the maximum amount of remaining variability (26,27). The data from each bacterial group were transformed into the arc cos square root of the percentage of that particular bacterial group. Extraction of the initial set of uncorrelated components was accomplished with the principal factor method and then the orthogonal rotation of components was used to facilitate interpretation. Factor loading was used to interpret the factor structure. Loadings are equivalent to Pearson correlation coefficients, and a higher loading indicates a stronger relation between a factor and an observed variable (27). Strong loading was defined as a value ≥ 0.6, and marginal loading as a value from 0.2 to 0.4. To evaluate the bacterial variables that were independently related to feeding modality (BF vs FF), we performed a binary logistic regression. All of the analyses were performed using the statistical package SPSS (SPSS Inc, Chicago, IL).

RESULTS

Fecal samples from healthy infants who had been exclusively BF or FF were collected at 12 weeks of age. The microbiota was analyzed by FISH-FC. In general, the predominant group detected was *Bifidobacterium*, followed by Enterobacteriaceae and *Bacteroides*. BF infants presented significantly lower proportions of *Atopobium* cluster ($P = 0.0001$), *Lactobacillus* group ($P = 0.004$), *C leptum* group ($P = 0.015$), and *Streptococcus* group ($P = 0.048$) compared with those of FF infants (Table 3). No significant differences were observed for the rest of the bacterial groups.

Regarding the principal component analysis results, 3 principal components were sufficient to explain the correlations among bacterial groups in both BF and FF infants. The 3 components explained 65.4% and 64% of the total variance in BF and FF infants, respectively. Those components were different for the BF and FF infants: *Bifidobacterium*/Enterobacteriaceae, *Lactobacillus*/*Bacteroides*, and *C coccoides*/*Atopobium* for BF infants (Table 4) and *Bifidobacterium*/*Enterobacteriaceae*, *Bacteroides*, and *C coccoides* for the FF infants (Table 5). The components were named according to the main group associated with BF or FF infant microbiota.

In BF infants, 29.9% of the variance was explained by the *Bifidobacterium*/Enterobacteriaceae component, 18.7% by the *Lactobacillus*/*Bacteroides* component, and 16.8% by the *C coccoides*/*Atopobium* component (Table 4). Similarly, in FF infants, the *Bifidobacterium*/Enterobacteriaceae component explained 29.2% of the variance, and 19.9% and 14.9% were explained by the *Bacteroides* and *C coccoides* components, respectively (Table 5).

Based on a binary logistic regression, we found that the *Lactobacillus* group was the only independent variable associated with fecal microbiota in FF infants (odds ratio 7.43, 95% confidence interval 2.00–27.64; $P = 0.003$).

DISCUSSION

In this study, the evaluation of the fecal microbiota using FISH-FC showed that the *Bifidobacterium* genus was the predominant group detected followed by Enterobacteriaceae and *Bacteroides* in both BF and FF infants. These results are in agreement with older data based on classic culture methods (13) and with those obtained recently by Turroni et al (28) and Yatsunenko et al (29), which revealed a predominance of bifidobacteria in the infant gut based on pyrosequencing data. Indeed, our data do not support the results of Palmer et al (14), who reported a low level of bifidobacteria in infant microbiota using 16S rRNA gene hybridization microarrays.

It is well known that bifidobacteria can be underrepresented when using genomic techniques for determination because DNA isolation can be affected by their thick cell walls (30). The paucity of bifidobacteria recently described can also be because of inefficient PCR analysis (28,31). Therefore, in the present study, we used FISH analysis, which does not require cell lysis, to detect bacteria with known DNA sequences with a sensitivity of $\geq 10^3$/g of feces (13). In fact, Fallani et al (1) used FISH-FC to show that the

TABLE 3. Comparison of the proportion of bacterial groups detected in fecal samples of breast-fed or formula-fed infants by fluorescent in situ hybridization and flow cytometry with a panel of 10 oligonucleotide probes

	Breast-fed infant, n = 31		Formula-fed infant, n = 27	
Targeted groups	Mean	SEM	Mean	SEM
Bifidobacterium genus (Bif164)	58.2	5.7	48.4	3.9
Enterobacteriaceae (Enter1432)	22.0	5.4	12.9	2.5
Streptococcus group (Strc493)	0.8	0.5	2.1*	0.4
Clostridium perfringens (Cpef191) + *Clostridium difficile* (Cdif198)	4.3	0.7	6.3	1.0
Clostridium leptum group (Clep1156)	0.5	0.2	2.4*	0.7
Lactobacillus group (Lab158)	0.5	0.1	2.3*	0.6
Bacteroides group (Bac303)	12.6	2.8	17.9	2.6
Atopobium cluster (Ato291)	0.3	0.1	7.7**	1.8
Clostridium coccoides group (Erec482)	0.9	0.9	0.1	0.0

SEM = standard error of the mean.
* Significant difference at $P < 0.05$.
** Significant difference at $P < 0.001$.

TABLE 4. Principal component analysis of breast-fed infants according to bacterial groups, genus, and species, as detected by fluorescent in situ hybridization and flow cytometry

	Component		
Targeted groups	*Bifidobacterium*/ Enterobacteriaceae	*Lactobacillus*/ *Bacteroides*	*Clostridium coccoides/Atopobium*
Bifidobacterium genus (Bif164)	−0.9	0.2	—
Enterobacteriaceae (Enter1432)	0.9	—	—
Streptococcus group (Strc493)	0.6	0.5	—
Clostridium perfringens (Cpef191) + *Clostridium difficile* (Cdif198)	0.5	0.3	−0.2
Clostridium leptum group (Clep1156)	0.2	—	—
Lactobacillus group (Lab158)	—	0.8	−0.3
Bacteroides group (Bac303)	—	−0.6	−0.2
Atopobium cluster (Ato291)	—	0.6	0.8
Clostridium coccoides group (Erec482)	—	—	0.9
Percent			
Variance	29.9	18.7	16.8
Cumulative proportion of variance	29.9	48.6	65.4

Results are expressed in loading factors. Factor loading is the product-moment correlation between an observed variable and an underlying factor. Strong loading was defined as a value ≥0.6 and marginal loading as a value from 0.2 to 0.4.

intestinal microbiota of 6-week-old BF infants had significantly higher proportions of *Bifidobacterium* genus and lower proportions of *Bacteroides, C coccoides*, and the *Lactobacillus* group compared with that of FF infants. Similarly, we found lower proportions of the *Lactobacillus* group in the BF infants; however, we also found significant differences in the proportions of the *Atopobium* cluster, *C leptum*, and the *Streptococcus* group between BF and FF infants, with a higher difference for the *Atopobium* cluster. Moreover, other studies have also shown that fecal samples of FF infants harbor higher numbers of the *Atopobium* cluster compared with BF infants (32,33). In addition, the number of clostridia found in BF infants is lower and has been considered as the only bacterial group that can be predictive for FF infants (34). Indeed, Fallani et al described *C coccoides* as a potential indicator group for FF infants (1). Thus, we found a higher proportion of *C leptum* in FF infants; however, in our study, the only independent bacterial group associated with FF infants was the *Lactobacillus* group.

Based on our study, the low frequencies of the *Atopobium* cluster, *C leptum, Lactobacillus* group, and the *Streptococcus* group in feces may explain the lower incidence of diseases in BF infants. Studies based on smaller infant groups identified bifidobacteria associated with protection from allergy development (35,36), whereas *Clostridium* spp, including *C difficile*, were associated with increased risk (35–37); however, a large study found no association between colonization by any particular bacterial group and development of atopic eczema or specific IgE to food antigens (38). Our group has previously shown that infants with cow's-milk protein allergy exhibit significantly higher numbers of the *C coccoides* group and the *Atopobium* cluster in their gut microbiota (39).

TABLE 5. Principal component analysis of formula-fed infants according to bacterial groups, genus, and species, as detected by fluorescent in situ hybridization and flow cytometry

	Component		
Targeted groups	*Bifidobacterium*/ Enterobacteriaceae	*Bacteroides*	*Clostridium coccoides*
Bifidobacterium genus (Bif164)	−0.8	−0.5	—
Enterobacteriaceae (Enter1432)	0.8	−0.2	−0.3
Clostridium leptum group (Clep1156)	0.7	—	—
Lactobacillus group (Lab158)	0.5	−0.5	0.3
Streptococcus group (Strc493)	0.5	—	0.6
Clostridium coccoides group (Erec482)	−0.3	—	0.7
Clostridium perfringens (Cpef191) + *Clostridium difficile* (Cdif198)	0.3	−0.4	0.6
Bacteroides group (Bac303)	—	0.8	—
Atopobium cluster (Ato291)	—	0.6	—
Percent			
Variance	29.2	19.9	14.9
Cumulative proportion of variance	29.2	49.1	64

Results are expressed in loading factors. Factor loading is the product-moment correlation between an observed variable and an underlying factor. Strong loading was defined as a value ≥0.6 and marginal loading as a value from 0.2 to 0.4.

Another main finding of this study was the identification of 3 main components that explain the major part of the variability of an infant's intestinal microbiota. Microbiota of BF and FF infants at the age of 12 weeks can be primarily described by the *Bifidobacterium*/Enterobacteriaceae component. According to this component, a BF infant harboring high levels of *Bifidobacterium* genus will also have low levels of the Enterobacteriaceae and *Streptococcus* group. In this regard, Tsuji et al (40) have reported that *Bifidobacterium* population levels were negatively correlated with those of Enterobacteriaceae in healthy Japanese infants. Although in FF infants the first component is also named *Bifidobacterium/*Enterobacteriaceae, high levels of *Bifidobacterium* genus have indicated low levels of Enterobacteriaceae and *C leptum.*

As mentioned before, *Bifidobacterium* have been associated with protection against allergy. Indeed, low levels of *Bifidobacterium* have been associated with allergic status (41–43), but other studies have found no association (44,45). In addition, a reduction of *Bifidobacterium* diversity has also been associated with allergy (46); however, the literature shows numerous discrepancies related to the link between bifidobacterial species and allergy (47). Accordingly, Waligora-Dupriet et al (47) showed that the diversity in *Bifidobacterium* colonization was not related to allergic status in both allergic and nonallergic French infants. Moreover, they showed that the link between *Bifidobacterium* colonization and allergy-based diseases is complex and cannot be restricted to the role attributed to *Bifidobacterium* species (47). Hence, we hypothesized that the high levels of *Bifidobacterium* genus and the lower levels of the Enterobacteriaceae and *Streptococcus* group in BF infants, compared with those found in FF infants, may contribute to the lower prevalence of allergy in the former.

The second component shows that harboring low levels of *Bacteroides* indicates higher levels of the *Lactobacillus* group and *Atopobium* cluster in BF infants. Recently, Jost et al (48) reported that neonates, exclusively BF, harboring high levels of *Bifidobacterium* show lower levels of *Bacteroides* and vice versa. According to our results, BF infants with low levels of *Bacteroides* also have high levels of *Bifidobacterium* genus, but with a marginal loading factor (0.2). Differences between the 2 studies may exist because they studied only 7 children. In FF infants, the *Bacteroides* component shows that harboring high levels of the *Bacteroides* group also indicates the presence of high levels of *Atopobium* cluster. Likewise, in FF infants, there was a weak negative correlation between *Bifidobacterium* genus and *Bacteroides.*

Clostridia are generally recognized to be more prevalent in FF than in BF infants (13). The last component, *C coccoides/ Atopobium*, reveals the positive association of *Atopobium* cluster and *C coccoides* in BF infants. Nevertheless, in FF infants, the *C coccoides* component indicated that higher proportions of the C *coccoides* group are associated with higher proportions of *C perfringens* plus *C difficile*, and the *Streptococcus* group.

Our study has some limitations. Although FISH-FC technology allows the detection of living bacteria with good sensitivity, we only studied 10 bacterial groups, genus, and species, whereas many other species are known to be present in human microbiota. Approximately 150 common species have recently been identified in human feces associated with 3 enterotypes (49,50).

In conclusion, we found a differential clustering of bacterial components for the infant gut microbiota based on the feeding method. Dysregulation of these bacterial clusters may be associated with disease. Further studies are clearly needed in this field to identify the main strains for each cluster and to determine the association of the clusters to specific diseases.

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