Intestinal Microbiota of 6-week-old Infants Across Europe: Geographic Influence Beyond Delivery Mode, Breast-feeding, and Antibiotics

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ABSTRACT

Objectives: There are many differences in diet and lifestyle across Europe that may influence the development of the infant gut microbiota. This work aimed to assess the impact of geographic area, mode of delivery, feeding method, and antibiotic treatment on the fecal microbiota of infants from 5 European countries with different lifestyle characteristics: Sweden, Scotland, Germany, Italy, and Spain.

Patients and Methods: Fecal samples from 606 infants (age 6 weeks) recruited within the European project INFABIO were analyzed by fluorescent in situ hybridization combined with flow cytometry using a panel of 10 rRNA targeted group- and species-specific oligonucleotide probes. Information on factors potentially affecting gut microbiota composition was collected with questionnaires and associations were evaluated with multivariate analyses.

Results: The *Bifidobacterium* genus was predominant (40% average proportion of total detectable bacteria), followed by *Bacteroides* (11.4%) and enterobacteria (7.5%). Northern European countries were associated with higher proportions of bifidobacteria in infant feces, whereas a more diverse microbiota with more bacteroides characterized southern countries. Bifidobacteria dominated the microbiota of breast-fed infants, whereas formula-fed babies had significantly higher proportions of *Bacteroides* and members of the *Clostridium coccoides* and *Lactobacillus* groups. Newborns delivered by cesarean section or from mothers treated with antibiotics perinatally had lower proportions of *Bacteroides* and members of the *Atopobium* cluster.

Conclusions: Delivery mode and feeding method influenced the fecal microbiota of European infants at 6 weeks, as expected, but the effect of country of birth was more pronounced, with dominant bifidobacteria in northern countries and greater early diversification in southern European countries.

Key Words: 16S rRNA probes, flow cytometry, fluorescent in situ hybridization, geography, infant fecal microbiota, infant feeding

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The human intestinal microbiota is a complex ecosystem, consisting of several hundred different bacterial species. This microbiota plays an important role in human health and nutrition by producing nutrients, preventing colonization of the gut by potential pathogenic microorganisms (1), and preserving the health of the host through interactions with the developing immune system (2). The microbiota in early life has been linked to allergy risk (3) and infection. A more recent study (4) suggested that the composition of the infant gut microbiota may be related to later risk of obesity.

Major changes in the intestinal microbial composition occur in early life. Sterile in utero, the gastrointestinal tract of the newborn infant is rapidly colonized at birth by a myriad of maternal vaginal and fecal bacteria and other sources from its environment (5). The first few weeks after birth correspond to critical stages of gut colonization. Bacterial colonization of the gastrointestinal tract is influenced by numerous factors including diet, environment, antibiotic treatment, mucosal maturation, and age.

Characterization of the microbiota represents a first step in understanding the contribution of the microbial community inhabiting the gastrointestinal tract to human physiology (6). Most previous studies, which generated our current understanding of gut microbiology and ecology of infants, relied almost exclusively on the use of culturing methods. However, these methods are not applicable to large sets of samples and are limited by their inability to detect noncultivable bacteria, representing 60% to 70% of all bacteria (7,8). New techniques based on bacterial RNA and DNA have been developed for investigating, identifying, and quantifying the intestinal microbiota. Molecular tools have mainly targeted ribosomal RNA and, more specifically, 16S rRNA (9). Fluorescent in situ hybridization (FISH) combined with flow cytometry (FC) is a particularly high-throughput method based on 16S rRNA probe hybridization, reliable for characterizing the composition of fecal microbiota in epidemiological studies (10–12).

In the past few years, several studies based on culture-independent techniques have investigated the molecular composition of the infant intestinal microbiota. These studies based on FISH (2,13), denaturing gel gradient electrophoresis (14), quantitative real-time polymerase chain reaction (15–17), or terminal-restriction fragment length polymorphism (18) showed a high variability in the first year of life of infants, depending on their mode of delivery, age, time of weaning, and feeding method, with particularly high numbers of bifidobacteria and *Bacteroides* and presence of lactobacilli/enterococci and coliforms. However, most of these studies have so far been restricted to small cohorts of infants and all with infants recruited within a single geographic region or country.

Given the increasing number of studies comparing infants from different countries to investigate the role of bacteria in the development of conditions such as allergy, it is important to understand the impact of country of birth on the development of the microbiota in infants without allergy. Large-scale molecular studies have compared the gut microbiota compositions of adults (19) and elderly adults (20) across Europe. The present work was undertaken to determine, using FISH-FC, the fecal microbiota composition of 606 young infants from 5 European countries with different lifestyle characteristics and to correlate this composition to country of origin, mode of delivery, feeding method, and perinatal antibiotic treatment.

PATIENTS AND METHODS

Sample and Data Collection

Fecal samples were available from 606 infants participating in the Diet and Environment longitudinal study of the European project INFABIO (*www.gla.ac.uk/infabio*). Infants were recruited in 5 different European centers: 158 from Glasgow (UK), 125 from Reggio Emilia (Italy), 116 from Stockholm (Sweden), 109 from Granada (Spain), and 98 from Düsseldorf (Germany). Mothers of newborn infants filled in a first questionnaire recording events concerning pregnancy and delivery. Once the baby was 6 weeks old, mothers filled in a second questionnaire concerning feeding method, health history of the baby at approximately 6 weeks, antibiotic treatment of infants or breast-feeding mother, and so forth.

Fecal Samples and Cell Fixation

Each fecal specimen, collected from the 606 infants at approximately 6 weeks of age, was placed in a sterile plastic box and maintained under anaerobic conditions at 4°C using an anaerocultA (Merck, Nogent sur Marne, France) for a maximum of 4 hours before processing for cell fixation as previously described (21). There was much discussion in the planning of the project of how to keep the conditions of collection and processing of the samples as standardized as possible but allowing for local conditions and practices at each center. Thus, soon after fecal samples were passed, they were placed into the anaerobic jar and maintained under anaerobic conditions and processed for cell fixation within 4 hours of passage. In some cases, research staff collected the sample in the diaper from the infant's home as soon as parents contacted the laboratory. In others, parents delivered the samples under anaerobic conditions quickly to the laboratory, or the infant provided the sample at the clinic, and this was placed in an anaerobic jar and sent to the laboratory and processed in the same way and time frame as all of the other samples. Sample fixation kits were provided to each collection center. Feces were homogenized by mechanical kneading for 3 minutes and aliquots of 1 g (wet weight) were added to 9 mL of anaerobic phosphate-buffered saline (PBS). The suspension was mixed to complete homogeneity in a 50-mL stoppered sterile glass jar and aliquots of 0.2 mL of the suspension were added to 0.6 mL of 4% paraformaldehyde (PFA) in PBS. After 1 night at 4°C, suspensions fixed in PFA were stored at −70°C and shipped on dry ice approximately every 3 months for analysis at a single location (INRA, Jouy en Josas, France).

FISH Method

As described previously (10,11), 400 μL of fixed suspension was mixed with 600 μL of PBS. Before hybridization, cells were always pelleted at 8000 *g* for 3 minutes in a microcentrifuge tube and resuspended in a volume of 1 mL. After washing in Tris-EDTA buffer (100 mmol/L Tris-HCl pH 8.0, 50 mmol/L EDTA pH 8.0), pellets were suspended in Tris-EDTA buffer containing 1 mg/mL of lysozyme (Serva, Heidelberg, Germany) and incubated for 10 minutes at room temperature. Cells were then washed in PBS to remove lysozyme and equilibrated in the hybridization solution (900 mmol/L NaCl, 20 mmol/L Tris-HCl pH 8.0, 0.01% sodium dodecyl sulfate pH 7.2, 30% formamide). A 50-μL aliquot of this suspension was used for FISH with control and group-specific probes. Hybridization was performed in a 96-well microtiter plate overnight at 35°C in the hybridization solution containing 4 ng/μL (final concentration) of the appropriate labeled probes. Following hybridization, 150 μL of hybridization solution was added in each well and cells were pelleted at 4000 *g* for 15 minutes. Washing was performed to remove nonspecific binding of the probe by incubating the bacterial cells at 37°C for 20 minutes in the washing solution (64 mmol/L NaCl, 20 mmol/L Tris-HCl pH 8.0, 5 mmol/L EDTA pH 8.0, 0.01% sodium dodecyl sulfate pH 7.2). Cells were finally pelleted at 4000 *g* for 15 minutes and resuspended in PBS. An aliquot of 100 μL was added to 0.4 mL of FACS FLOW for FC detection.

Data Acquisition by Flow Cytometry

Data acquisition was performed with a FACS Calibur flow cytometer (Becton Dickinson, Erembodegem-Aalst, Belgium) equipped with an air-cooled argon ion laser providing 15 mW at 488 nm combined with a 635-nm red-diode laser, as described previously (10,11). All of the parameters were collected as logarithmic signals. The 488-nm laser was used to measure the forward angle light scatter (FSC, in the 488-nm band pass filter), the side angle light scatter (SSC, in the 488-nm band pass) and the green fluorescence intensity conferred by fluorescein isothiocyanate–labeled probes (FL1, in the 530-nm band pass filter). The red-diode laser was used to detect the red fluorescence conferred by

Cy5-labeled probes (FL4, in a 660-nm band pass filter). The acquisition threshold was set in the side scatter channel. The rate of events in the flow was generally below 3000 events per second. A total of 100,000 events were stored in list mode files for each sample. Subsequent analyses were conducted using the CellQuest Software (Becton Dickinson). Analyses were performed as described previously (22) by creating density plots and delineating regions to encompass double-labeled bacteria.

Oligonucletide Probes

The EUB 338 probe, conserved within the domain bacteria (23), was used as a positive control of hybridization. Conversely, the NON 338 probe (24) was used as a negative control. These oligonucleotide probes were covalently linked at their 5′ end either with FITC or with Cy5 (Thermo Electron, Ulm, Germany). 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 1) (25–31). Enumeration of the different bacterial groups or species was performed by FISH-FC by combining in the same tube 1 specific probe labeled with Cy5 together with the EUB 338 FITC probe.

Statistical Analysis

Data are expressed as average and standard deviation of the proportions of cells that hybridized with each of the 10 oligonucleotide probes relative to the total bacteria. A value of zero was used to calculate the means whenever a microbial group was undetected or detected below the threshold of sensitivity of 0.4% (25). A general linear model was used to investigate outcome variables of interest (proportions of bacterial groups detected) across other main effects (country effect, delivery method, antibiotic treatment on infant and on mother during pregnancy) while correcting for feeding method. We did not correct for socioeconomic status because this has not been suggested to influence the microbiota at 6 weeks beyond its impact on breast-feeding rates in some countries. The same model was used to compare the outcome variables of interest across the 3 different feeding methods (fully breast-fed, formula fed, mixed fed) while correcting for the country effect. Comparisons were made using the Bonferroni correction factor to compensate for multiple testing. The geographical distribution observed in the composition of dominant fecal microbiota was assessed using principal component analyses (PCAs) by comparing the principal components across country of birth using analysis of variance. All analyses were performed using Minitab (version 14, Minitab Ltd, Coventry, UK) with a significance level of 5%.

RESULTS

Infant Feeding Practice

The average proportion of fully breast-fed infants of the total of 606 infants considered was 51.5%, whereas the proportion of formula-fed infants was 30.1% and the proportion of mixed-fed infants was 18.4% of the total infants. During this study, some infant formula manufacturers began including prebiotics in infant formula. It was not the intention of this study to investigate the impact of prebiotics on the fecal microbiota. The infant formula fed to the infants in this study varied between infants and centers and it was not possible to identify for certain which infants had received prebiotics.

Assessment of the Microbiota Composition of Infant Fecal Samples With FISH Combined With Flow Cytometry

Nearly all of the samples received (98%) could be analyzed. There was insufficient fecal material in the other 2%. When the data of all 606 infants were considered together, the predominant group by far detected was *Bifidobacterium*, with 40% of all detectable bacteria (±30.6%), followed by *Bacteroides* (11.4% ± 17.6%) and Enterobacteria (7.5% ± 15.9%). The *Clostridium coccoides* group, the main predominant group of the adult gut microbiota, presented proportions of only 5.5% (±11.5%), whereas *Clostridium perfringens* + *Clostridium difficile* species represented 3% of the total (±8.4%). *Atopobium* cluster, *Streptococcus* group, and *Lactobacillus* group represented 2.1% ± 6.2%, 1.6% ± 3.5%, and 1.2% ± 4.0% of the total, respectively, whereas the *Clostridium leptum* group, also a majority in the adult microbiota, presented average proportions at the limit of detection of the method (0.4% ± 2.3%). When the proportions of the bacterial cells detected were added together, a mean of 72.7% ± 24.5% was obtained with the panel of 10 nonoverlapping phylogenetic probes.

Impact of Country of Birth

The proportions of the different bacterial groups detected in the different countries are given in Table 2. Looking at the different bacterial groups, the center of origin was found to have a high impact, particularly for bifidobacteria, *Bacteroides*, and enterobacteria. A lesser impact was observed for members of the *C coccoides*, *Lactobacillus*, and *Streptococcus* groups, whereas no difference between countries was found for the less represented *C leptum* group, the *Atopobium* cluster, and the *C difficile* and *C perfringens* species. Samples from infants born in Granada, where breast-feeding rate was

TABLE 1. Panel of probes used for in situ assessment of microbiota composition of feces of infants

Probes	Sequence from 5′ to 3′ end	Targeted groups	Reference
Bif164	CATCCGGCATTACCACCC	*Bifidobacterium* genus	(26)
Bac303	CCAATGTGGGGGACCTT	*Bacteroides* group	(27)
Enter1432	CTTTTGCAACCCACT	Enterobacteria	(28)
Strc493	GTTAGCCGTCCCTTTCTGG	*Streptococcus* group	(29)
Lab158	GGTATTAGCAYCTGTTTCCA	*Lactobacillus* group	(30)
Ato291	GGTCGGTCTCTCAACCC	*Atopobium* cluster	(31)
Erec482	GCTTCTTAGTCARGTACCG	*Clostridium coccoides* group	(29)
Clep866	GGTGGATWACTTATTGTG	*Clostridium leptum* group	(22)
Cpef191	GCTCCTTTGGTTGAATGATG	*Clostridium perfringens* spp	(25)
Cdif198	TCCATCCTGTACTGGCTCACC	*Clostridium difficile* spp	(25)

TABLE 2. Mean and standard deviation of the proportions of bacterial groups, genus, and species detected in 606 infants' fecal samples coming from 5 different European countries, assessed by fluorescent in situ hybridization combined with flow cytometry with a panel of 10 oligonucleotide probes

City of origin (country), number of individuals	*Bifidobacterium* genus (Bif164)	*Clostridium coccoides* group (Erec482)	*Clostridium leptum* group (Clep866)	*Atopobium* cluster (Ato291)	*Bacteroides* group (Bac303)	Enteric group (Enter1432)	*Lactobacillus* group (Lab158)	*Streptococcus* group (Strc493)	*Clostridium difficile* sp* (Cdif198)*	*Clostridium perfringens* sp (Cperf191)*	*Clostridium perfringens* + *Clostridium difficile* sp	Sum
Stockholm (Sweden), n = 116	59.9 ± 31.8	2.5 ± 8.5	0.2 ± 1.2	2.4 ± 7.3	6.0 ± 11.7	1.7 ± 5.2	0.5 ± 1.3	1.4 ± 2.3	1.0 ± 2.0	14.7 ± 12.1	3.2 ± 7.8	77.8 ± 25.3
Glasgow (UK), n = 158	40.2 ± 30.1	9.4 ± 14.9	0.2 ± 1.5	3.2 ± 7.8	10.5 ± 15.5	3.5 ± 8.2	0.4 ± 1.5	1.7 ± 4.4	1.0 ± 3.0	15.0 ± 25.2	4.5 ± 13.1	73.7 ± 24.6
Düsseldorf (Germany), n = 98	38.1 ± 26.1	7.5 ± 12.3	0.7 ± 3.3	2.2 ± 5.3	10.7 ± 15.1	6.5 ± 11.0	1.7 ± 5.1	1.3 ± 3.0	0.4 ± 1.6	11.7 ± 14.3	2.0 ± 5.3	70.6 ± 19.0
Reggio Emilia (Italy), n = 125	41.0 ± 26.1	5.0 ± 10.8	0.2 ± 0.5	1.7 ± 5.7	9.7 ± 15.9	8.3 ± 13.4	0.9 ± 1.9	2.4 ± 3.7	0.3 ± 0.9	9.7 ± 10.2	2.6 ± 5.6	71.6 ± 22.8
Granada (Spain), n = 109	18.9 ± 24.1	1.6 ± 3.7	1.0 ± 3.6	0.7 ± 2.0	21.3 ± 24.5	19.3 ± 27.8	2.8 ± 7.3	1.3 ± 2.9	0.9 ± 1.2	8.4 ± 1.5	2.2 ± 3.5	69.0 ± 28.5
Total, N = 606	40.0 ± 30.6	5.5 ± 11.5	0.4 ± 2.3	2.1 ± 6.2	11.4 ± 17.6	7.5 ± 15.9	1.2 ± 4.0	1.6 ± 3.5	0.7 ± 2.0	12.4 ± 16.1	3.0 ± 8.4	72.7 ± 24.5

Values indicate the percentage of cells hybridizing with the corresponding probe versus Eub338 probe (mean ± SD). For the sum we considered the combined values of *Clostridium perfringens* and *Clostridium difficile* species detected instead of the separate values.

*Cdif198 and Cperf191 used separately only on 46 samples chosen among the samples with the highest proportions detected for Cdif198 + Cperf191 in the different centers (9 in Düsseldorf, 12 in Glasgow, 5 in Granada, 10 in Reggio Emilia, and 10 in Stockholm). Target groups for each probe included (23) Bif164, *Bifidobacterium* genus; Erec482, species of *Clostridium*, *Eubacterium*, *Ruminococcus*, and *Butyribivrio* genera; Clep866, members of *Clostridium*, *Eubacterium*, *Ruminococcus*, and *Anaerofilum* genera + *Faecalibacterium prausnitzii* cluster; Ato291, *Atopobium* cluster including *Coriobacterium* genus; Bac303, *Prevotella* and *Bacteroides* genera; Enter1432, Enteric group including *Escherichia coli*; Lab158, *Lactobacillus*, *Enterococcus*, *Weissella*, and *Pediococcus* genera; Strc493, *Streptococcus* group including *Lactococcus* genus; Cdif198, *Clostridium difficile* sp; Cperf191, *Clostridium perfringens* sp.

43.1% at the time the samples were collected, presented significantly greater proportions of *Bacteroides* and enterobacteria, and significantly lower proportions of bifidobacteria, compared with all other countries (Table 2). Granada samples also presented lower proportions of *C coccoides* members compared with Glasgow ($P < 0.001$) and Düsseldorf ($P = 0.002$) and higher proportions of members of the *Lactobacillus* group compared with Glasgow ($P < 0.001$), Stockholm ($P = 0.003$), and Reggio Emilia ($P = 0.007$). On the contrary, Stockholm (breast-feeding rate 75.9%) presented significantly higher proportions of bifidobacteria compared with all of the other centers, Glasgow (breast-feeding rate 48.7%) showed higher proportions of members of the *C coccoides* group compared with all of the centers except Düsseldorf (breast-feeding rate 45%), whereas Reggio Emilia (breast-feeding rate 62.4%) had higher proportions of enterobacteria compared with Glasgow ($P = 0.029$) and Stockholm ($P = 0.012$) and higher proportions of members of the *Streptococcus* group compared with Glasgow ($P = 0.047$) and Düsseldorf ($P = 0.010$). No significant difference was found between centers for the sum of the proportions of the different bacterial groups analyzed. The geographic trend in microbiota composition was further supported by PCA (Fig. 1A). Infants' microbiota were distributed along the axes of the first and second principal components (accounting for 22% and 14% of variability, respectively) as a function of the country of birth with an apparent north–south distribution. The overall sum of detected bacterial groups best explained the principal component 1, and proportions of bifidobacteria and bacteroides were inversely associated along principal component 2 in such a way that northern countries were characterized by a preweaned infant fecal microbiota highly dominated by bifidobacteria, whereas southern countries showed more *Bacteroides* and the highest early diversification of infant microbiota. The possibility of a confounding effect of feeding method on the impact of country of birth was excluded by a PCA showing that infant microbiota in Stockholm (Fig. 1B) and Granada (Fig. 1C) did not cluster as a function of feeding method.

Impact of the Feeding Method

Comparisons of the different bacterial groups detected with FISH-FC across the three feeding methods (fully breast-feeding, formula feeding, and mixed feeding) were determined using a linear model while correcting for the country effect. The adjusted mean values for each feeding type are shown in Figure 2. Breast-fed infants presented significantly greater proportions of bifidobacteria (44.8% vs 29.9%, $P < 0.001$) and significantly lower proportions of *Bacteroides* (8.8% vs 15.9%, $P < 0.001$), *C coccoides* (3.7% vs 6.9%, $P = 0.014$), and *Lactobacillus* groups (0.9% vs 1.9%, $P = 0.046$) compared with formula-fed babies. Breast-fed infants also presented significantly lower proportions of *Bacteroides* compared with mixed-fed babies (8.8% vs 13.8%, $P = 0.034$), whereas proportions of bifidobacteria were still significantly higher in mixed-fed infants than in formula-fed (40.9% vs 29.9%, $P = 0.007$) infants. Finally, formula-fed babies also presented lower proportions of *C perfringens* species compared with breast-fed infants (4.6% vs 24.3%, $P = 0.006$), while no significant difference was observed for *C difficile* species alone and *C perfringens* and *C difficile* detected together.

Impact of the Delivery Method

The mode of delivery had an impact on some bacterial groups. The adjusted mean values of the different bacterial groups detected for each delivery method are shown in Figure 3. Compared with cesarean section, vaginal delivery (67% of the total) was

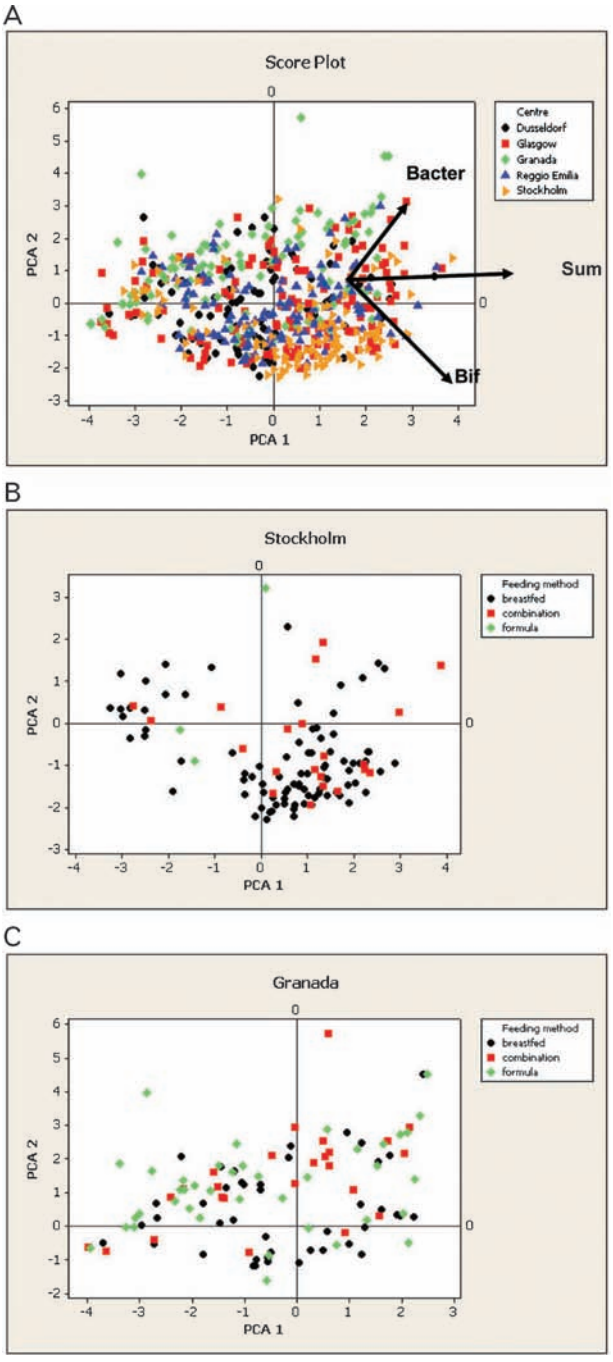

FIGURE 1. (A) Score plot of principal component analysis (PCA) showing the first 2 principal components explaining 22% (component PCA1, variance 2.62) and 14% (PCA 2, variance 1.67) of the variability, respectively. The geographical distribution of the first 3 principal components was assessed using analysis of variance on the PCA scores. For score 1, there was a significant geographical effect ($P < 0.001$), with Glasgow and Stockholm having higher values and Düsseldorf and Granada having lower values. Score 2 also showed a significant geographical effect ($P < 0.001$), with Granada having high scores and Stockholm having low scores. The effect was not significant for score 3 ($P = 0.084$). Arrows indicate the

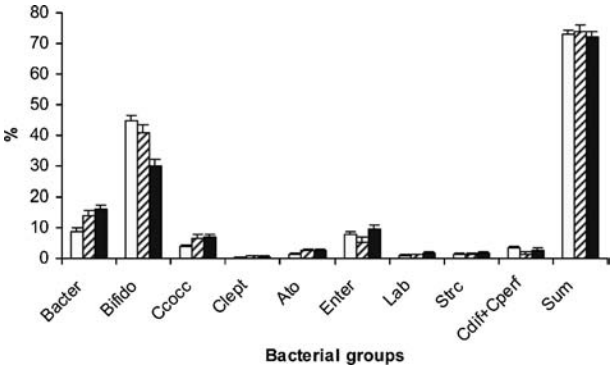

FIGURE 2. Composition of the fecal microbiota of preweaned infants (approximately 6 weeks of age) as a function of the feeding method (exclusively breast-feeding [□], mixed feeding [▨], and formula feeding [■]). Values are mean proportions of the bacterial groups quantified by FISH-FC. Bars indicate standard deviation. Stars indicate significant differences between diets ($^{*}P < 0.005$, $^{**}P < 0.001$). Codes for bacterial groups were Bacter, *Bacteroides* group; Bifido, *Bifidobacterium* genus; Ccocc, *Clostridium coccoides* group; Clept, *Clostridium leptum* group; Ato, *Atopobium* cluster; Enter, *Enterics* group; Lab, *Lactobacillus* group; Strc, *Streptococcus* group; C dif + C perf, *Clostridium difficile* + *Clostridium perfringens* species. FC = flow cytometry; FISH = fluorescent in situ hybridization.

associated with higher average proportions of *Bacteroides* (16.1% vs 6.9%, $P < 0.001$) and members of the *Atopobium* cluster (2.9% vs 0.8%, $P < 0.001$) and lower proportions of members of the *C coccoides* group (4.5% vs 8.2%, $P < 0.001$) and the *Streptococcus* group (1.4% vs 1.9%, $P = 0.048$). Vaginally delivered infants also presented a greater proportion for the sum of detected groups compared with the other babies (75.4 vs 67.6, $P < 0.001$). There was no effect of the mode of delivery on the relative proportions of bifidobacteria.

Effect of Antibiotic Treatments

Newborns who received antibiotics (only 7% of the 606 children investigated) presented significantly higher proportions of enterobacteria (16.6%) compared with those without treatment (6.8%) ($P < 0.001$). On the contrary, when mothers received antibiotic treatment perinatally and/or during breast-feeding, infants presented significantly lower average proportions of *Bacteroides* (11.4% vs 15.0%, $P = 0.029$) and members of the *Atopobium* cluster (1.5% vs 2.6%, $P = 0.044$), as well as for the total sum of detected

FIGURE 1. (*Continued*) projection of contribution of variables including Bacter (*Bacteroides* and relatives) Bif (*Bifidobacterium* genus) and Sum (additive contribution of the probes used, representative of the highest diversification for the lowest values). Score plot of PCA showing the first 2 principle components for the microbiota of preweaned infants (approximately 6 weeks) from Stockholm (Fig. 1B) and Granada (Fig. 1C) as a function of feeding mode. There was no significant clustering of microbiota as a function of mode of feeding.

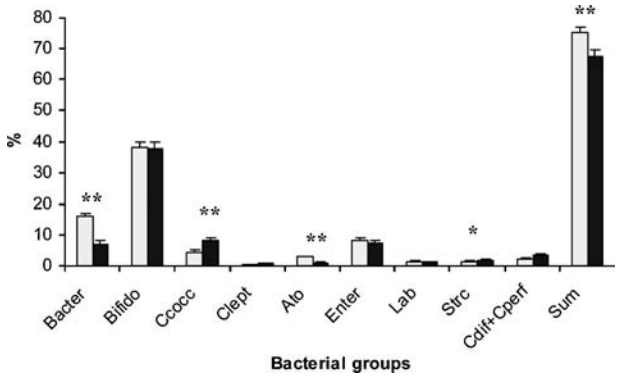

FIGURE 3. Composition of the fecal microbiota of preweaned infants (approximately 6 weeks of age) as a function of mode of delivery (vaginal [□] and cesarean section [■]). Values are mean proportions of the bacterial groups quantified by FISH-FC. Bars indicate standard deviation. Stars indicate statistically significant differences between delivery modes ($^{*}P < 0.005$, $^{**}P < 0.001$). FC = flow cytometry; FISH = fluorescent in situ hybridization. Codes for bacterial groups as in Figure 2.

groups (69.6% vs 76.1%, $P = 0.005$), compared with those whose mother received no treatment during pregnancy. There was a wide range of antibiotics received by individual infants and mothers and differences between countries. Many mothers could not identify which antibiotic was used. It was, therefore, not possible to carry out any detailed analysis.

DISCUSSION

The aim of this study was to assess the gut microbiota composition of the young European infant by analyzing 606 fecal samples obtained from babies at approximately 6 weeks from 5 countries with different lifestyle characteristics. The present study also investigated the impact of some important variables such as geographic origin, feeding method, mode of delivery, and antibiotic treatment on the early development of the intestinal microbiota of children. There are many differences in diet and lifestyle characteristics across Europe. For example, in Scotland, breast-feeding rates are lower (32) and many infants are weaned before 3 months (33). In contrast, in Scandinavian countries, breast-feeding rates are high and infants are weaned later (34,35). One of the main ways that diet and environment influence the infant is through their effects on the gut microbiota and its metabolism (36), which may have important effects on the health of the infant and later on the longer-term health of the child and adult.

In this study, we confirmed previously published work (13–17,37–39) that bifidobacteria are the predominant group detected in the feces of preweaned infants, followed by *Bacteroides* and enterobacteria. More recent studies based on molecular approaches also confirmed these findings (13–17,40–42). Dore et al (40) detected rRNA proportions of 30% to 40% for bifidobacteria in 2-month-old babies by dot-blot hybridization, whereas Martin et al (41) found a proportion of 21.7% of bifidobacteria and 34.6% of enterobacteria in preweaning fecal samples. Harmsen et al (13) found that infants aged 20 days harbored a microbiota in which bifidobacteria was predominant (30%–80%), particularly in breast-fed infants, but *Bacteroides* were also important (20%–60%), particularly in formula-fed babies, while *Escherichia coli* represented around 5% to 10% of the total.

A strong impact of the geographic origin was observed in the present study. This effect was particularly observed for *Bacteroides*, bifidobacteria, and enterobacteria. Our observations suggest a possible ''geographic gradient'' in the composition of the gut microbiota in Europe, where the extremes, north (Glasgow and Stockholm) and south (Granada and Reggio Emilia), would present the highest number of differences. It should be noted, however, that the centers from which the infants were recruited in the present study may not be representative of the country in which they were based because only 1 center was used in each case.

The north–south gradient was characterized by higher proportions of bifidobacteria, *Atopobium*, *C perfringens* + *C difficile*, and sum of total detectable bacteria in north European countries, and by higher proportions of *Bacteroides*, enterobacteria, and lactobacilli in south European countries, whereas *C coccoides*, *C leptum*, and streptococci remained unaffected by the country of birth at the age considered. To our knowledge, this is the first cross-sectional study comparing the impact of country of origin on the development of the gut microbiota of babies born in different European countries. A few previous studies have compared the microbiota composition for 2 countries and the majority considered infants born in developing countries as well (42,43). Sepp et al (44) reported high counts of lactobacilli and eubacteria in Estonian infants and increased numbers of clostridia in Swedish babies, with bifidobacteria and anaerobic cocci equally prevailing in both groups, which they related to risk of allergy. It is well established that in Western industrialized countries, routine hygienic procedures aimed at reducing the spread of bacteria in maternity and neonatal wards have strongly influenced the colonization pattern of newborn infants, whereas infants born in developing countries are exposed to a heavier bacterial load from birth and this condition influences the colonization pattern of the gut. The low colonization rate of enterobacteria in infants born in Swedish hospitals, reported by Lundequist et al (38), is probably related to these practices. Meanwhile, 2 recent studies investigated cross-sectional differences (19,20) in microbiota in young and older adults across Europe. In young adults (19), no significant differences with respect to geographic origin were found, but the research included only volunteers from central to northern European countries (Denmark, United Kingdom, the Netherlands, France, Germany). In the other study (20), which compared young adults and elderly adult volunteers from Sweden, France, Germany, and Italy, a significant difference was observed for the *Bifidobacterium* group, with the population in Italy having 2- to 3-fold higher proportions of bifidobacteria than that in any other country. However, the factors that determine the complex and relatively stable microbiota of adult populations, in which diet and other lifestyle factors may vary considerably, may be different from those that influence the early colonization of the infant gut at 6 weeks of age.

The geographic origin was far more important for the composition of the preweaned infant microbiota than any other parameter. Yet, in accordance with previous culture-based studies (37,38,45–47) and more recent molecular studies (13,16,41), we observed an important impact of the feeding method on the early development of the infant gut microbiota. Breast milk, even in mixed feeding, clearly favored bifidobacteria, whereas in its absence, a more diversified microbiota was established, with higher proportions of *Bacteroides* and members of the *C coccoides* and *Lactobacillus* groups. Comparing several individual studies, Tannock (47) found that numbers of clostridia were always lower in breast-fed babies and that clostridia was the only group predictive of formula feeding. Our observations agreed with this concept, focusing on *C coccoides* as a potential indicator group.

The mode of delivery also significantly influenced the microbiota composition of the newborns' intestine. Upon vaginal delivery,

the infant is predominantly exposed to vagina and fecal bacteria of maternal origin. Conversely, infants born by cesarean delivery have an initial exposure to environmental bacteria from equipment, air, other infants, and nursing staff. Vaginally delivered babies presented higher proportions of *Bacteroides* and members of the *Atopobium*, as well as added proportions of detectable bacteria, and lower proportions of members of the *C coccoides* and the *Streptococcus* groups compared with those born by cesarean section. Interestingly, the latter showed the same trend as infants born to mothers who received antibiotics during late pregnancy and/or while breast-feeding. A previous study (48) reported a considerable delay in the establishment of a stable microbiota in infants born by cesarean section, characterized by a low incidence of *Bacteroides* spp and a low isolation rate of other bacteria. Gronlund et al (49) also reported a delay in fecal colonization and a low number of *Bacteroides fragilis* in cesarean-delivered infants. In a recent study, Penders et al (16), investigating the fecal microbiota of 1032 Dutch infants by quantitative real-time polymerase chain reaction, observed that infants born by cesarean section had lower numbers of *Bacteroides* and bifidobacteria and were more often colonized with *C difficile* than were vaginally born infants. Hence, antibiotic treatment and cesarean delivery may promote the same suboptimal development of the microbiota in early infancy.

Concerning detection of *C difficile* and *C perfringens* species, an average of $3.0\% \pm 8.4\%$ was found with the 2 probes coupled together, whereas separate detection of the 2 species on 46 samples, chosen among those with high combined counts, showed 0.7% and 12.4% average proportions, respectively. *C perfringens* was formerly detected within 2 days of life (39) and differences concerning these species were observed, using selective culture media, as a function of the feeding method (17,37,39), mode of delivery (50), and geographic origin (45). In the present study, no differences were observed for *C difficile* and *C perfringens* species detected together for any of the variables investigated. Only breast-fed babies were found to have significantly higher proportions of *C perfringens* species compared with formula-fed infants ($P = 0.006$, 24.3% vs 4.6%), but for a subset of the samples investigated.

In conclusion, in this large-scale study, we highlighted the impact of geographic origin, feeding method, delivery mode, and antibiotic treatment on the composition of the fecal microbiota of European infants at 6 weeks of age. Above all, the colonic microbiota of the young healthy infant appeared different across Europe. The potential of a south-to-north gradient among the European countries investigated needs to be researched further to establish the gradient using more centers and to determine the factors responsible.

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