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Clostridium difficile Colonization in Early Infancy Is Accompanied by Changes in Intestinal Microbiota Composition[▽]

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***Clostridium difficile* is a major enteric pathogen responsible for antibiotic-associated diarrhea. Host susceptibility to *C. difficile* infections results partly from inability of the intestinal microbiota to resist *C. difficile* colonization. During early infancy, asymptomatic colonization by *C. difficile* is common and the intestinal microbiota shows low complexity. Thus, we investigated the potential relationship between the microbiota composition and the implantation of *C. difficile* in infant gut. Fecal samples from 53 infants, ages 0 to 13 months, 27 negative and 26 positive for *C. difficile*, were studied. Dominant microbiota profiles were assessed by PCR-temporal temperature gradient gel electrophoresis (TTGE). Bacterial signatures of the intestinal microbiota associated with colonization by *C. difficile* were deciphered using principal component analysis (PCA). Resulting bands of interest in TTGE profiles were excised, sequenced, and analyzed by nucleotide BLAST (NCBI). While global biodiversity was not affected, interclass PCA on instrumental variables highlighted significant differences in dominant bacterial species between *C. difficile*-colonized and noncolonized infants ($P = 0.017$). Four bands were specifically associated with the presence or absence of *C. difficile*: 16S rRNA gene sequences related to *Ruminococcus gnavus* and *Klebsiella pneumoniae* for colonized infants and to *Bifidobacterium longum* for noncolonized infants. We demonstrated that the presence of *C. difficile* in the intestinal microbiota of infants was associated with changes in this ecosystem's composition. These results suggest that the composition of the gut microbiota might be crucial in the colonization process, although the chronology of events remains to be determined.**

Clostridium difficile is the most common cause of antibiotic-associated diarrhea and pseudomembranous colitis in adults. *C. difficile* infections (CDI) are increasing and are mainly linked to the use of wide-spectrum antibiotics that disrupt the intestinal microbiota equilibrium. This allows *C. difficile* to multiply and colonize the gut, this being the first step in the pathogenic process (2, 31, 34). *C. difficile* then produces its toxins, TcdA and TcdB, mediating cell damage and clinical signs (3). Colonization is an essential step in the pathogenic process of *C. difficile* that depends, on the one hand, on *C. difficile* colonization factors and, on the other hand, on the microbiota colonization resistance (barrier effect). However, other host factors, such as the immune response of the host, may also participate in this step (23). The loss of the commensal microbiota barrier effect and the release of ecological niches previously unavailable following antibiotic treatment allow *C. difficile* of endogenous or exogenous origin to colonize the gut. Recent studies suggest that restoration of the microbiota by the use of bacteriotherapy (probiotic use and fecal transplantation) is accompanied by resolution of patients' symptoms (13, 17). Intestinal microbiota composition appears to play a central role in induction of disease and relapse of CDI.

To explain the host susceptibility to CDI and recurrences, it is strongly suggested that the commensal microbiota could be more or less permissive to the establishment of *C. difficile*. Indeed, recent work highlighted that microbiota composition, assessed before antibiotic treatment, differed between individuals who developed CDI as a consequence and those who did not (7). Chang et al. showed a decreased fecal microbiota diversity in adults with recurrent CDI (4). Thus, the composition of the intestinal microbiota could play a role as a predisposing factor in the onset of the disease.

In early infancy, asymptomatic carriage of *C. difficile* in the digestive tract is very common. Many infants are colonized by toxigenic or nontoxigenic *C. difficile* strains during the first two years of life (5). This colonization is rarely associated with CDI. Fecal microbiota is less complex in infants under 2 years of age than in adults (12). During the first months, a particularly high number of bifidobacteria is observed in breast-fed babies. After 6 months, *Bacteroides*, the *Clostridium coccoides* group, and *Faecalibacterium prausnitzii* become detectable in increasing amounts (10, 15). However, the *Firmicutes*/*Bacteroidetes* ratio is lower than in adults (22). Fallani et al. detected *C. difficile* in infants older than 5 months (11). They also observed that infants with detectable proportions of *C. difficile* had lower percentages of bifidobacteria and higher proportions of *Bacteroides*.

The aim of the present work was to look for bacterial signatures associated with *C. difficile* colonization status in the infant intestinal microbiota using a molecular approach coupled with powerful statistical analysis. This work would help in understanding the process of colonization by *C. difficile* in

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infants. Determinants of the microbiota composition associated with *C. difficile* colonization status give information about bacterial groups involved in the barrier effect against *C. difficile* implantation. These results pave the way for defining targeted strategies for microbiota modulation with an anti-*C. difficile* objective.

MATERIALS AND METHODS

Subjects and samples. A systematic screening of *C. difficile* in fecal samples from infants of ages 0 to 13 months in the pediatric ward or pediatric emergency unit or being hospitalized was performed at the University Hospital Jean Verdier. One stool sample was collected per infant from the diapers or after defecation. For the study, 27 positive samples and 26 samples negative for the presence of *C. difficile* were selected (infants were age matched). Exclusion criteria were defined as follows: history of antibiotic use within the previous 4 weeks, diarrhea (defined as at least 3 loose stools per day with no consistency) (25), documented infectious gastroenteritis, intravenous feeding, severe illness, immunosuppression, and bowel surgery. Several aliquots were prepared from each sample and either stored at +4°C for *C. difficile* detection or frozen at −80°C into sterile Starstedt 2.2-ml screw cap tubes for molecular analyses. The presence of *C. difficile* in fecal specimens was screened by toxigenic culture on fresh stool, considered a reference method combining a selective culture and toxin detection, as previously described (27). For each *C. difficile* isolate, genes encoding toxins were screened by PCR using the method of Lemée et al. for *tcdA* and *tcdB* (encoding toxins A and B) and that of Stubbs et al. for *cdtA* and *cdtB* (encoding binary toxin) (18, 32).

In addition, a kinetic study was conducted with four healthy infants during their first year of life. For each infant, one stool sample was collected every month. Samples were treated following the above-described procedure. The protocols for the present study were approved by the Evaluation Committee of Ethics of Biomedical Research Projects (CEERB) of the Northern University Hospital Group of Paris (no. 09-005).

Clinical and environmental data. Clinical and environmental data were collected for each infant that took account of information contained in the medical chart, the hospital database, and the clinician's or parents' information. Items included clinical and environmental data that might impact the microbiota composition: age at collection of fecal sample, gender, term of birth, mode of delivery (vaginal delivery or caesarean), type of feeding (exclusively breast fed, exclusively formula fed, combination of breast and formula fed, or diversified), and atopic history (infant, parents, or siblings). Data on factors that may influence *C. difficile* intestinal implantation were also collected: use of antacids for gastroesophageal reflux, unit of consultation/hospitalization, and stay duration when applicable.

DNA isolation and 16S rRNA gene amplification. Total DNA was extracted from 200 mg of fecal samples as previously described (29, 33). DNA concentration and integrity were determined both visually by electrophoresis on a 1% agarose gel containing ethidium bromide and spectrophotometrically by using a Nanodrop instrument (Thermo Scientific). The primers GCclamp-U968 (5′ CGC CCG GGG CGC GGC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TAC) and L1401 (5′ GCG TGT GTA CAA GAC CC) were used to amplify the V6 to V8 regions of the bacterial 16S rRNA genes. Amplification by PCR was performed as previously described (19, 29, 33). PCR products were analyzed by electrophoresis on a 1% agarose gel containing ethidium bromide to check for appropriate sizes of the amplicons.

TTGE fingerprinting. The Dcode universal mutation detection system (Bio-Rad, Paris, France) was used to separate PCR products according to their specific sequence (GC percentage) by temporal temperature gradient gel electrophoresis (TTGE). Electrophoresis was performed as described earlier (19, 29). Gels were stained with SYBR green I nucleic acid gel stain (Roche Diagnostics, GmbH, Mannheim, Germany) and read on the Storm system (Molecular Dynamics).

Obtained TTGE profiles were analyzed with the GelCompar software program (version 2.2; Applied Maths, Kortrigk, Belgium). The analysis took into account the number of bands, their positions on the gel, and their intensities. This software translates each TTGE profile into a densitometric curve, drawing a peak for each band whose area is proportional to the optical density of the band. A marker consisting of a PCR amplicon mix of 7 cloned rRNA genes from different bacterial species was used to normalize the profiles. This marker, loaded at 3 positions on each gel (each side and middle), allows correction of any distortion in the gel migration and comparison of profiles between gels. Similarity coefficients (Pearson correlation method) were calculated for each pair of profiles, yielding a similarity matrix. A dendrogram was constructed from this matrix by using an unweighted pair group method using arithmetic averages (UPGMA) algorithm (19). Statistical analyses were performed for interindividual comparisons using the chi-square test and the Mann-Whitney test (Wilcoxon).

Statistical analysis of dominant microbiota profiles. Densitometric curves corresponding to each of the normalized TTGE profiles were digitized with the GelCompar software program from the migration distances ranging from 1 to 383 at steps of 1 interval and the observed optical density at each distance step. The resulting data matrix was used to calculate the spatial coordinates of each individual within a principal component analysis (PCA) using multivariate regression. PCAs were computed with the R software program (6) (package ade4; http://pbil.univ-lyon1.fr/ADE-4/) to document the distances between dominant microbiota composition of all the infants' feces. In order to decipher the impact of the different environmental factors on microbiota composition, interclass PCAs with different clinical and environmental characteristics as instrumental variables were computed based on the presence and abundance of each profile's bands for each individual. Interclass PCA with instrumental variables allows highlighting combinations of variables (here, TTGE bands representing dominant bacterial species) that maximize variations observed between qualitative variables (e.g., *C. difficile* colonization status). *P* values of the statistical significance of interclass PCA clustering based on microbiota profiles were assessed using a Monte Carlo rank test (999 replicates). Interclass PCA with instrumental variables was further applied to decipher the most discriminating variables (TTGE bands) between microbiota of infants colonized or not by *C. difficile*. The relevance of these bands in terms of intensity and frequency was checked on the data matrix.

TTGE band extraction and sequencing. Each band of interest (as statistically determined by interclass PCA with instrumental variables) was extracted from TTGE gels of fecal samples of two different subjects. Gel fragments were incubated in diffusion buffer (EDTA [2 mM], sodium acetate [0.3 M] [pH 7.5 to 7.8]) (mass/vol) at 50°C for 30 min and centrifuged for 1 min at 12,000 × *g*. The collected supernatant was treated using the QIAquick gel extraction kit (Qiagen), following the manufacturer's procedure. rRNA gene fragments were then reamplified by PCR as described above. Following PCR purification (GeneJet PCR purification kit; Fermentas), amplicon size was checked on 1.5% agarose gels containing ethidium bromide and the concentration was spectrophotometrically evaluated (Nanodrop). Each PCR product was sequenced (both forward and reverse) using the Sanger methodology. Obtained sequences were compared to public available 16S rRNA gene sequences by nucleotide BLAST (GenBank, NCBI).

RESULTS

To determine whether changes occurred in the intestinal dominant species composition according to *C. difficile* colonization in infants, we compared fecal microbiota associated with the presence or absence of *C. difficile*. Fifty-three infants were selected for the study, 27 with negative *C. difficile* culture and 26 with positive *C. difficile* culture on fecal samples. Among the 26 *C. difficile* carriers, 10 were colonized with a toxigenic strain.

Age was taken into account for homogeneity in the repartition, which was as follows: 0 to 2 months ($n = 18$), 2 to 5 months ($n = 18$), and 5 to 13 months ($n = 17$). In age groups 0 to 2, 2 to 5, and 5 to 13 months, *C. difficile* colonized/noncolonized infants accounted, respectively, for 6/12, 10/8, and 10/7 infants. Most of the infants were sampled during the first 48 h of admission (46/53) and did not receive antacid treatment (47/53).

In addition, the four infants kinetically followed during their first year of life acquired *C. difficile* between the 3rd and 5th months and remained colonized. Fecal samples obtained before ($n = 2$) and during ($n = 2$) *C. difficile* colonization were analyzed by TTGE.

Dominant fecal microbiota diversity. All TTGE profiles of infant fecal samples displayed a relatively low complexity, with a mean number of bands of 13 ± 4. The biodiversity was not modified by the presence of *C. difficile* in the fecal microbiota.

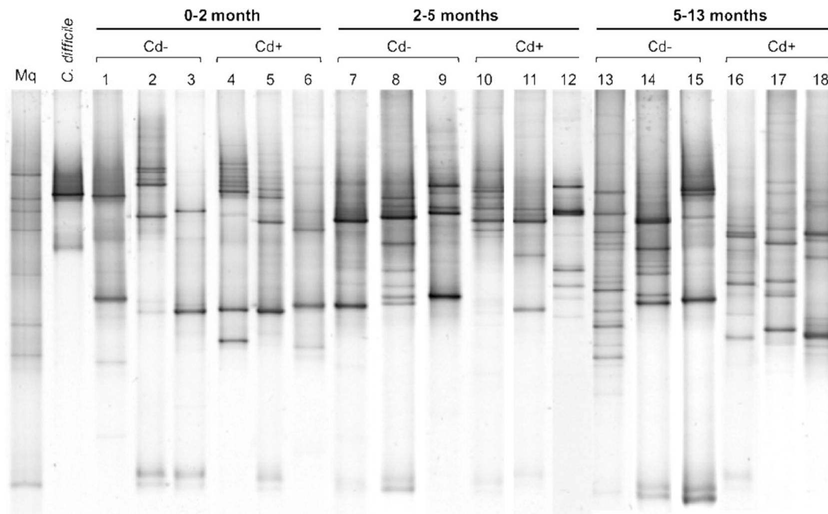


FIG. 1. TTGE fingerprinting profiles of 16S rRNA gene amplicons (obtained using primers for the V6 to V8 regions) of a *C. difficile* strain and 18 fecal samples from <1- to 13-month-old infants (lanes 1 to 18). Mq, marker consisting of a PCR amplicon mix of 7 cloned rRNA genes from different bacterial species; Cd+, positive *C. difficile* culture; Cd−, negative *C. difficile* culture.

The mean number of bands was 14 ± 4 for *C. difficile*-colonized infants and 13 ± 4 for non-*C. difficile*-colonized infants ($P = 0.168$). TTGE banding patterns from 18 of the infants are shown in Fig. 1. The 53 independent TTGE profiles were compared in a single dendrogram that did not show any clustering according to presence or absence of *C. difficile* (data not shown).

Principal component analysis of the dominant microbiota. The PCA on fingerprint TTGE profiles of dominant intestinal bacterial species from the 53 infants (Fig. 2A) highlighted a large dispersion, especially between noncolonized infants, and no *C. difficile*-associated specific microbiota profile could be identified among the infants. An interclass PCA with instrumental variables was performed using colonization status by *C. difficile* as a variable, with a distinction between toxigenic and nontoxigenic strains (Fig. 2B). The Monte Carlo rank test showed no significant difference among the 3 groups: (i) infants colonized with a toxigenic strain, (ii) infants colonized with a nontoxigenic strain, and (iii) infants not colonized ($P = 0.418$). The toxin effect on microbiota composition was analyzed, and the rank test showed that no specific dominant microbiota profile was associated with colonization by a toxigenic strain ($P = 0.584$). However, the presence of *C. difficile* itself in the intestinal microbiota was significantly associated with modifications in the intestinal microbial composition ($P = 0.017$).

Since other factors might influence the microbiota composition in early infancy (25, 26), interclass PCAs were realized on a set of potential confounder variables, and results of the Monte Carlo rank tests are shown in Table 1. No significant differences were observed for most of these variables. Parameters that influence microbiota profiles were age of the infants ($P = 0.001$), type of feeding ($P = 0.022$), and term of birth ($P = 0.034$).

In order to overcome the effect of confounding variables, a homogeneous subgroup of infants was selected and TTGE profiles of the 20 infants of ages 2 to 5 months were analyzed. No significant differences were found between patterns according to age ($P = 0.216$), term of birth ($P = 0.188$), and feeding ($P = 0.294$) within this subgroup. However, microbiota profiles still differed significantly between *C. difficile*-colonized and noncolonized groups (Monte Carlo $P = 0.020$) (Fig. 2C).

For the 4 infants monitored over time, PCA was carried out on TTGE profiles obtained from fecal samples collected between the ages of 2 and 6 months, when colonization by *C. difficile* occurred. Interclass PCAs with age, subject, and *C. difficile* colonization as instrumental variables showed no significant difference according to age ($P = 0.128$) in this subgroup, whereas *C. difficile* colonization status was significantly associated with modifications in the microbiota composition ($P = 0.012$) (Fig. 2D).

Bacterial species associated with *C. difficile* colonization status. Statistical analysis of the discriminating variables between infants colonized or not colonized by *C. difficile* highlighted 3 TTGE bands associated with *C. difficile*-colonized infants' profiles (Cd^+ bands) and 4 bands with non-*C. difficile*-colonized infants' profiles (Cd^- bands). The same specific bands were identified in the subgroup of infants of ages 2 to 5 months. Bands' positions on the gels and samples that were analyzed are shown in Fig. 3. Cd^+ bands corresponded to 16S rRNA gene sequences related to *Klebsiella pneumoniae* (band 2), *Ruminococcus gnavus* (band 3), and a relative of *Clostridium nexile* (band 4). Cd^- bands corresponding 16S rRNA gene sequences corresponded to *Staphylococcus epidermidis* (band 1), *Escherichia coli* (band 5), and *Bifidobacterium longum* subsp. *longum* bv. infantis (bands 6 and 7), as presented in Table 2.

Frequencies and intensities of these specific bands were determined on both densitometric curves and gel scans (Fig. 4). All 26 *C. difficile*-colonized infants had at least one Cd^+ band, 11 had all the Cd^+ bands (42.3%), and 25 had 2 or 3 Cd^+ bands (96.2%). Three of these infants also presented Cd^- bands. Among the 27 non-*C. difficile*-colonized infants, 25 had at least one Cd^- band and 20 had 2 or 3 Cd^- bands (74.7%).

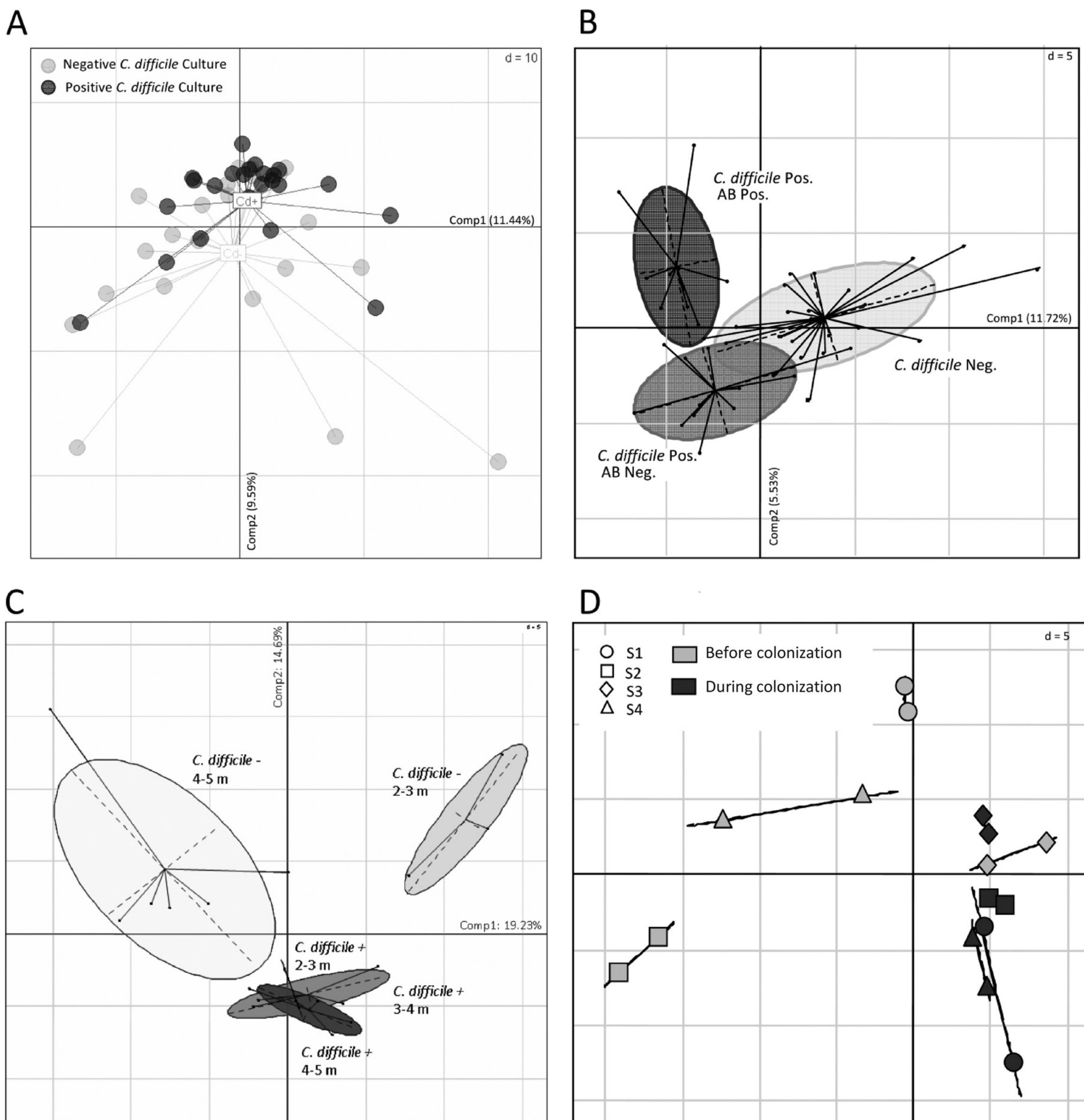


FIG. 2. Principal component analysis of TTGE fingerprinting profiles of dominant intestinal bacterial species (fecal samples). (A) PCA of TTGE profiles of 53 infants of ages <1 to 13 months. Black circles represent infants colonized by *C. difficile*, and light-gray circles represent noncolonized infants. (B, C, and D) Interclass PCA of TTGE profiles with instrumental variables. Individuals (represented by black dots or symbols) were clustered (ellipses or bars), and the center of gravity was computed for each class. (B) Interclass PCA of TTGE profiles of 53 infants of ages <1 to 13 months, with *C. difficile* toxigenic culture status as an instrumental variable. The global Monte Carlo test showed no significant difference between the 3 groups ($P = 0.418$). Intestinal colonization by a toxigenic strain of *C. difficile* was not associated with a specific TTGE profile ($P = 0.584$). Colonization by *C. difficile* was significantly associated with modifications in the microbiota composition ($P = 0.017$). (C) Interclass PCA of TTGE profiles of 20 infants of ages 2 to 5 months, with *C. difficile* culture and age as instrumental variables. Based on a Monte Carlo test with 999 replicates, a significant difference was found between all groups ($P = 0.003$). Intestinal colonization by *C. difficile* was significantly associated with a specific TTGE profile ($P = 0.02$). The age group of the infants was not associated with modifications in the microbiota composition ($P = 0.125$). (D) Interclass PCA of TTGE profiles of 4 infants of ages 2 to 6 months, with subject and *C. difficile* culture as instrumental variables. Samples were obtained before ($n = 2$) and during ($n = 2$) colonization by *C. difficile.* One sample collected during colonization was excluded from the analysis for infant S4 as carried out under antibiotic therapy. Intestinal colonization by *C. difficile* and subject itself were significantly associated with modifications in the microbiota composition as assessed by a Monte Carlo test (respectively, $P = 0.012$ and $P = 0.041$). Pos., positive; Neg., negative; AB, toxin A/B. *C. difficile* −, negative *C. difficile* culture. *C. difficile* +, positive *C. difficile* culture; Comp, component; m, month(s); S, subject.

Eight of these infants also presented Cd^+ bands. Median band intensities were not significantly different except for *R. gnavus*, which was significantly more abundant in association with *C. difficile*.

The temporal relevance of these specific bacterial species was assessed before and during colonization by *C. difficile* for the 4 monitored infants. In this context, bands statistically associated with *C. difficile* intestinal implantation corresponded

TABLE 1. Characteristics of infants and Monte Carlo rank tests on interclass PCAs with clinical and environmental features as instrumental variables of TTGE profiles

Characteristic	No. of infants per group[a]	Monte Carlo simulated P value[b]
Age	18 (<1-2 mo), 18 (>2-5 mo), 17 (>5-13 mo)	0.001
C. difficile culture	27 Cd^-, 26 Cd^+	0.017
Feeding	7 breast, 17 For, 11 mixed, 11 Div, 3 NA	0.022
Term of birth	8 preterm, 42 term, 2 NA	0.034
Season	3 Win 08, 17 Sum 08, 27 Win 09, 6 Sum 09	0.150
Delivery mode	37 vaginal delivery, 7 caesarean, 11 NA	0.282
Site	18 outpatients, 31 pediatrics, 4 neonatalogy	0.527
TcdA, TcdB	16 A^-B^-, 10 A^+B^+	0.584
Atopy	18 $atopy^-$, 19 $atopy^+$, 16 NA	0.721
Gender	25 F, 28 M	0.978

[a] NA, not available; Cd^-, negative *C. difficile* culture; Cd^+, positive *C. difficile* culture; For, formula milk; mixed, breast and formula milk; Div, diversified feeding; Win, winter; Sum, summer; F, female; M, male; 08, 2008; 09, 2009.

[b] Monte Carlo rank test calculates the simulated P value by extrapolating the test (hypothesis: significant difference between TTGE profiles of the populations) with 999 replicates. Significant differences are observed between TTGE profiles for $P < 0.05$.

to *R. gnavus* (band 3) and *K. pneumoniae* (band 2). For the 4 infants, the *R. gnavus* band was exclusively found during colonization. The *K. pneumoniae* band was specifically found during colonization in 3 of the infants. Prior to *C. difficile* colonization, only the 2 bands corresponding to *B. longum* were observed in 3 infants.

DISCUSSION

Although numerous studies of microbial invasion of the human gut have been performed and fluctuation of the fecal populations in babies has been well established, little is known about *C. difficile* implantation in this ecosystem. Asymptomatic carriage of *C. difficile* is frequent in infants up to 2 years old (5). In the study of Fallani et al., *C. difficile* was specifically detected by fluorescent *in situ* hybridization (FISH) combined with flow cytometry and accounted for 0.5% ± 1% of the total microbiota (11). The bacterium was more frequently detected in infants older than 5 months. However, until now no study of the infant intestinal microbiota has been performed that specifically takes into account asymptomatic colonization by *C. difficile.* Thus, we analyzed by molecular profiling the fecal microbiota of infants colonized and not colonized by *C. difficile* in order to highlight differences in the dominant microbiota according to *C. difficile* implantation. Based on the comparison of nucleic acid sequences of the 16S rRNA genes, TTGE fingerprinting allows the characterization of approximately 90% of the dominant fecal microbiota species (9, 36). This technique is also very efficient for monitoring the evolution of bacterial populations over time or environmental changes (19).

In the present study, TTGE profiles presented a large dispersion, underlining an important interindividual variability in infant microbiota, and no *C. difficile*-associated specific intestinal profile could be identified. The species diversity was not modified by the presence of *C. difficile* in the fecal microbiota. This is in line with a previous study from Chang et al., where the authors showed that the diversity of adult fecal microbiota, as assessed by a molecular inventory, was identical in control and CDI patients. Only patients with recurrent CDI presented a significant biodiversity decrease. The authors

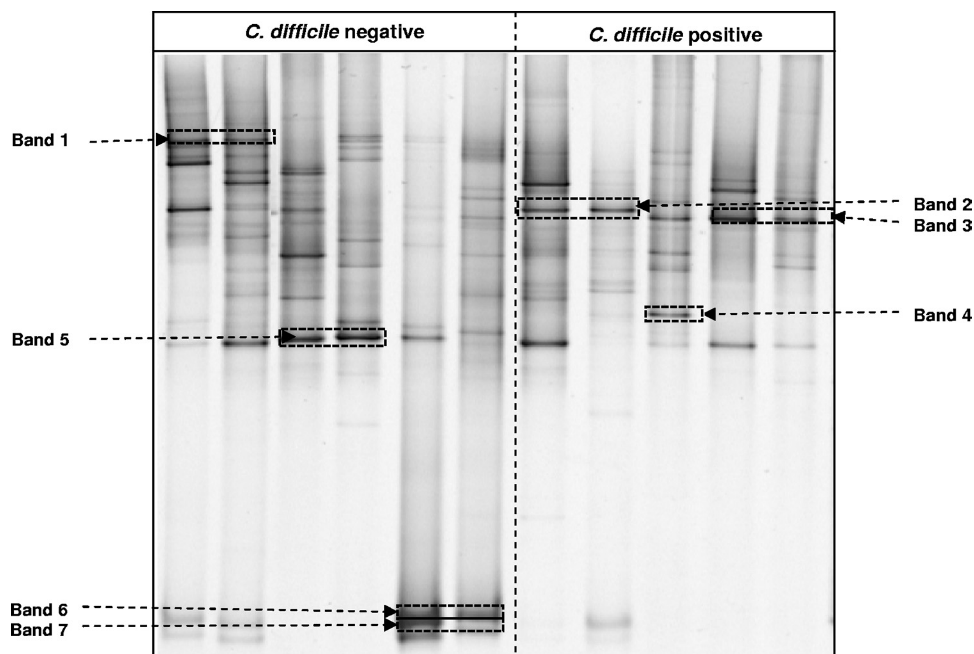


FIG. 3. TTGE profiles of 16S rRNA gene amplicons (obtained using primers for V6 to V8 regions) of fecal samples and localization of bands associated with colonization or noncolonization by *C. difficile*.

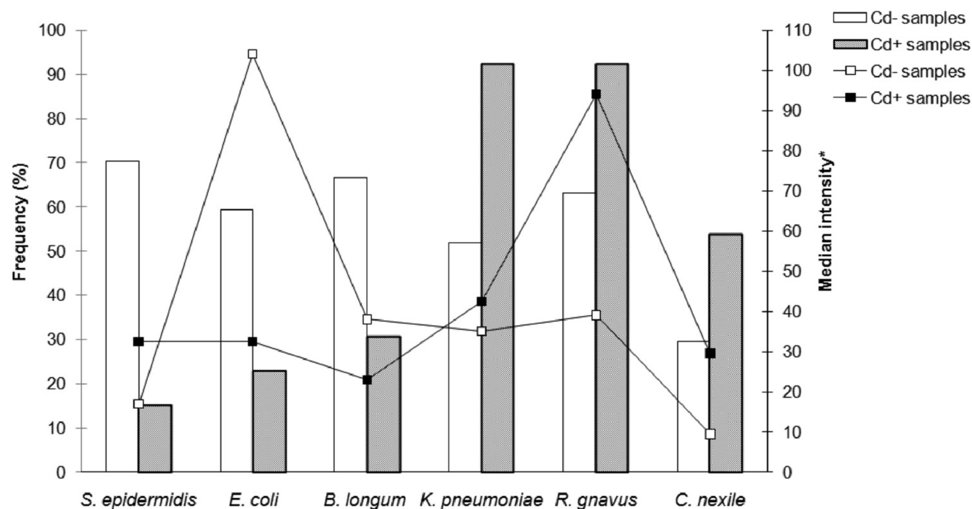
TABLE 2. Bands specifically associated with *C. difficile* colonization or noncolonization in infants[a]

Band	Sample	1st BLAST hit	Id (%)	Closest isolate relative	Id (%)
1	CR09-095	Uncultured bacterium clone nbw620h07c1	99	*Staphylococcus epidermidis*	99
	CR09-073	Uncultured bacterium partial 16S rRNA gene, clone MA01A05	98	*Staphylococcus epidermidis*	98
2	CR08-029	*Klebsiella pneumoniae* strain SDM45	99		
	CR08-044	*Klebsiella pneumoniae* strain SDM45	99		
3	Ma4	Uncultured bacterium clone PM8_a04h01	99	*Ruminococcus gnavus*	99
	CR08-036	Uncultured bacterium clone S3-210	99	*Ruminococcus gnavus* strain A2	98
4[b]	CR08-052	Uncultured bacterium clone 3-7D8	100	*Clostridium nexile* DSM 1787	94
5	CR08-020	*Escherichia coli* strain A34	99		
	CR08-015	*Escherichia coli* strain A_R2A6	99		
6	CR08-014	*Bifidobacterium longum* subsp. *longum* strain R0175	100		
	CR08-069	Uncultured bacterium clone C4-189	99	*Bifidobacterium longum* bv. *infantis* strain KLDS 2.0611	99
7	CR08-014	*Bifidobacterium longum* subsp. *longum* strain R0175	99		
	CR08-069	Uncultured bacterium clone C4-189	99	*Bifidobacterium longum* bv. *infantis* strain KLDS 2.0611	99

[a] Characteristics and best sequence isolate hit match using nucleotide BLAST are given. Id, identity.
[b] Two samples were used for band excision and consecutive DNA extraction on the TTGE profile, except for band 4 due to the failure of DNA extraction from one of the two samples.

suggested that the observed altered microbiota was deficient in the ability to restore colonization resistance against *C. difficile* (4).

In the present work, we demonstrated that the presence of *C. difficile* in the intestinal microbiota, whatever the strain toxigenicity, was significantly associated with modifications in the microbial ecosystem composition. During early infancy, multiple factors have been identified as potentially influencing the composition of the intestinal microbiota (24, 25, 26, 28). As expected, in our study parameters that influenced dominant bacterial composition were age of the infants, type of feeding, and term of birth. A PCA on a homogeneous subgroup of infants of ages 2 to 5 months not influenced by these factors confirmed the link between the microbiota composition and the presence of *C. difficile*. Furthermore, the monitoring of four infants during the colonization process also showed that



	Cd- samples (n = 27)			Cd+ samples (n = 26)				
	Frequency (%) (n)	Median intensity*	SD	Frequency (%) (n)	Median intensity*	SD	Frequency P-value‡	Median P-value†
S. epidermidis	70.4 (19)	17,0	20.9	15.4 (4)	32.5	21.5	0.0002	0.29
E. coli	59.3 (16)	104,0	43.6	23.1 (6)	32.5	64.1	0.0167	0.32
B. longum	66.7 (18)	38,0	46.7	30.8 (8)	23,0	33.3	0.0194	0.39
K. pneumoniae	51.9 (14)	35,0	25.5	92.3 (24)	42.5	33.3	0.0030	0.67
R. gnavus	63.0 (17)	39,0	32.4	92.3 (24)	94,0	45.3	0.0262	0.01
C. nexile	29.6 (8)	9.5	12,0	53.8 (15)	29.5	52.4	0.1311	0.11

FIG. 4. Frequency and median intensity of TTGE profile bands corresponding to bacterial species associated with presence or absence of *C. difficile* in infant feces. Median intensity, curves; frequency, bars; Cd−, negative *C. difficile* culture; Cd+: positive *C. difficile* culture; SD, standard deviation. *, median intensity is expressed in absolute units. ‡, chi square test on frequency; †, Mann Whitney U test (Wilcoxon) on median intensity.

the implantation of *C. difficile* was associated with significant changes in the microbiota composition.

It is likely that the modifications associated with the implantation of *C. difficile* involve several bacterial species, and we were able to identify species that discriminated between the colonized and noncolonized infants' microbiota. Three bacterial species were identified both for a broad set of infants and during the colonization monitoring of four infants. *Bifidobacterium longum* was associated with fecal microbiota of non-*C. difficile*-colonized infants, while colonized infants more frequently presented the *Ruminococcus gnavus* and *Klebsiella pneumoniae* species. Moreover, *R. gnavus* appeared to be in higher proportions in colonized infants. Three other species might play a role in the intestinal colonization by *C. difficile* but were not recovered by the intraindividual study. *Staphylococcus epidermidis* and *Escherichia coli* were preferentially found in noncolonized infants, and a relative of *Clostridium nexile* was found in colonized infants.

In recent studies in adults, several bacterial groups have been suggested as being associated with protection against *C. difficile* colonization. Hopkins and Macfarlane analyzed the fecal microbiota in elderly subjects and were able to demonstrate that the bacterial species diversity was markedly lower in CDI patients than in healthy controls (14). In CDI patients, the microbiota was characterized by a high number of facultative anaerobes and low levels of *Bifidobacterium* and *Bacteroides* (14). A central role for *Bacteroides* spp. in colonization resistance against *C. difficile* has also been suggested by others (20). Fallani et al. showed that children with detectable proportions of *C. difficile* had lower proportions of bifidobacteria and higher proportions of *Bacteroides* (11). In our study, the *Bifidobacterium* spp. also appear as a characteristic of the noncolonized infants. Interestingly, a recent study highlighted that the proportions of *Bifidobacterium* species in early fecal samples of young infants significantly correlated with the total levels of salivary secretory IgA at 6 months old (30). On the other hand, studies using anti-immunoglobulin antibodies showed significant reductions in IgA-producing cells in CDI biopsy specimens ($P < 0.05$), with the greatest reduction in samples from patients with pseudomembranous colitis. Johal et al. concluded that a selective reduction in mucosal IgA-producing cells and macrophages is associated with colonic disease in *C. difficile*-infected patients and that a severe reduction in colonic IgA-producing cells may predispose to a recurrence of CDI (16). Many studies have shown that the presence of *C. difficile* or CDI is associated with decreased levels of bifidobacteria. Our results are consistent with these previous works, and all of this raised the possibility that bifidobacteria play a protective role against *C. difficile* colonization, potentially involving mucosal immunity of the gut.

Our results showed that the colonized infant group was characterized by a higher frequency of detection of *Klebsiella pneumoniae*, a facultative anaerobic bacterium naturally resistant to aminopenicillins. In the work of Hopkins and Macfarlane, patients with CDI had elevated fecal levels of facultative anaerobes (14). During antibiotic-associated CDI, the disruption of the intestinal microbiota homeostasis by antibiotics precedes the multiplication of *C. difficile*. Several studies showed that amoxicillin-clavulanic acid treatment led to a dramatic decrease in butyrate-producing bacteria, such as the *Eubacterium rectale-Clostridium coccoides* group, and an increase in the *Bacteroidetes* and the *Enterobacteriaceae* (1, 31, 35). This disequilibrium induced by antibiotics could facilitate intestinal colonization by *C. difficile*. In the absence of antibiotics to disrupt the microbiota, it is not clear which event precedes the other between colonization by *C. difficile* and microbiota modifications. However, in infants a low *Firmicutes*/*Bacteroidetes* ratio and an increase in facultative anaerobes might facilitate colonization by *C. difficile* without a need for the action of antibiotics.

R. gnavus and to a lesser extent a species related to *C. nexile* were associated with the presence of *C. difficile*. These two species have been shown to produce a trypsin-dependent antimicrobial substance against *Clostridium perfringens* but with a lesser activity against *C. difficile* (21). Moreover, *C. nexile* has previously been reported to be stimulated upon antibiotic chemotherapy (8).

In conclusion, our study gives some clues to the microbiota composition allowing *C. difficile* colonization. The presence in important quantities of a *Firmicutes* species such as *R. gnavus* is associated with *C. difficile* colonization. In contrast, the presence of *Bifidobacterium* spp. appears to be related to an absence of *C. difficile* colonization and might participate in the colonization resistance properties of the infant gut microbiota, which potentially involves mucosal immunity. However, these first results need to be confirmed in a larger-scale study with different time points to decipher which phenomenon precedes the other. Quantitative variations could also be specified in targeting preferentially the species identified in this study. *C. difficile* colonization is likely a result of an imbalanced ecosystem in the gut. A cocktail of probiotics might help restore intestinal microbiota composition.

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