Clostridium difficile Colonization in Early Infancy is Accompanied by Changes in the Intestinal Microbiota Composition.

Running Title: Clostridium difficile impact on infant gut microbiota

Keywords: Clostridium difficile, intestinal microbiota, TTGE, colonization

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ABSTRACT

_Clostridium difficile_ is a major enteric pathogen responsible for antibiotic-associated diarrhea. Host susceptibility to _C. difficile_ infections partly results from the intestinal microbiota inability to resist to _C. difficile_ colonization. During early infancy, asymptomatic colonization by _C. difficile_ is common and 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 of 53 infants, 27 negative and 26 positive for _C. difficile_, aged 0 to 13 months 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, inter-class PCA on instrumental variables highlighted significant differences in dominant bacterial species between _C. difficile_ colonized and non-colonized infants (p=0.017). Four bands were specifically associated to the presence or absence of _C. difficile_: 16S rDNA sequences related to _Ruminococcus gnavus_ and _Klebsiella pneumoniae_ for colonized infants and to _Bifidobacterium longum_ for non-colonized 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.
INTRODUCTION

Clostridium difficile is the most common cause of antibiotic-associated diarrhea and pseudomembranous colitis in adults. C. difficile infections (CDI) are increasing and 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 of 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 (12, 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, a recent work highlighted that microbiota composition, assessed before antibiotic treatment, differed between individuals who developed CDI as a consequence as compared to those who did not (6). 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 predisposing factor to 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 non-toxigenic *C. difficile* strains during the two first years of life (5). This colonization is rarely associated with CDI. Fecal microbiota is less complex in infants under two years of age than in adulthood (11). During the first months, a particularly high number of bifidobacteria is observed in breast-fed babies. After 6 months *Bacteroides*, *Clostridium coccoides* group and *Faecalibacterium prausnitzii* become detectable in increasing amounts (9, 14). However, the ratio *Firmicutes/Bacteroidetes* is lower than in adults (22). Fallani *et al.* detected *C. difficile* in infants older than 5 months (10). 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 a powerful statistical analysis. This work would help understanding the colonization process by *C. difficile* in infants. Determinants of the microbiota composition associated with *C. difficile* colonization status will give information about bacterial groups involved in the barrier effect against *C. difficile* implantation. These results pave the way to define 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 aged 0 to 13 months consulting in the pediatric ward, 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 negative samples for *C. difficile* presence were selected (infants 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. Presence of *C. difficile* in fecal specimens was screened by toxigenic culture on fresh stool, considered as a reference method combining a selective culture and toxin detection, as previously described (27). On each *C. difficile* isolate, genes encoding toxins were screened by polymerase chain reaction (PCR) using the method of Lemée *et al.* for *tcdA* and *tcdB* (Toxin A and B) and Stubbs *et al.* for *cdtA* and *cdtB* (binary toxin) (18, 32).

In addition, a kinetic study was conducted on 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 (n° 09-005).

**Clinical and environmental data**

Clinical and environmental data were collected for each infant that take 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 anti-acids for gastro-oesophageal reflux, unit of consultation / hospitalization and stay duration when applicable.

**DNA isolation and 16S rDNA 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 Nanodrop (Thermo Scientific). The primers GCclamp-U968 (5’ CGC CCG GGG CGC GGC CCG GGC 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 rDNA. 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 size of the amplicons.

**Temporal Temperature Gradient Gel Electrophoresis Fingerprinting**

The Dcode Universal Mutation Detection System (Bio-Rad, Paris, France) was used to separate PCR products according to their specific sequence (GC percent) 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 GelCompar software (version 2.2 Applied Maths, Kortrijk, Belgium). The analysis took into account the number of bands, their position on the gel
and their intensity. This software translates each TTGE profile into a densitometric curve, drawing a peak for each band which area is proportional to the optical density of the band. A marker consisting of a PCR amplicon mix of 7 cloned rDNAs 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 inter-individual comparisons using Chi-square test and Mann-Whitney test (Wilcoxon).

**Statistical analysis of dominant microbiota profiles**

Densitometric curves corresponding to each of the normalized TTGE profiles were digitized with GelCompar software 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 R software (8) (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, inter-class 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. Inter-class 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 inter-class PCA clustering based on microbiota profiles were assessed using a Monte-Carlo rank test (999 replicates). Inter-class 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. Relevance of these bands in terms of intensity and frequency was checked on the data matrix.

**TTGE bands extraction and sequencing**

Each band of interest (as statistically determined by inter-class 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 7.5-7.8) m/v at 50°C for 30 min and centrifuged for 1 min at 12,000g. The collected supernatant was treated with the QIAquick Gel Extraction Kit (QIAGEN) following manufacturer’s procedure. rDNA gene fragments were then re-amplified 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 concentration was spectrophotometrically evaluated (Nanodrop). Each PCR product was sequenced both forward and reverse using Sanger methodology. Obtained sequences were compared to public available 16S rDNA 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 0-2, 2-5 and 5-13 months, *C. difficile* colonized/non-colonized infants accounted respectively for 6/12, 10/8 and 10/7. Most of the infants have been sampled during the first 48 hours of admission (46/53), and did not receive anti-acid treatment (47/53).

In addition, the four infants kinetically followed during their first year of life acquired *C. difficile* between the 3rd and 5th month 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. Mean number of bands was 14±4 for *C. difficile* colonized infants and 13±4 for *C. difficile* non-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 of the 53 infants (Fig. 2A) highlighted a large dispersion, especially between non-colonized infants, and no *C. difficile* associated specific microbiota profile could be identified within the infants. An inter-
class PCA with instrumental variables was performed using colonization status by C. difficile as variable, with distinction between toxigenic and non-toxigenic strains (Fig. 2B). The rank test of Monte-Carlo showed no significant difference among the 3 groups: i) infants colonized with a toxigenic strain, ii) infants colonized with a non-toxigenic strain and iii) infants not colonized (p=0.418). 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, presence of C. difficile itself in the intestinal microbiota was significantly associated with modifications in the intestinal microbial composition (p=0.017).

As other factors might influence the microbiota composition in early infancy (25, 26), inter-class 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 sub-group of infants was selected and TTGE profiles of the 20 infants aged 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 sub-group. However, microbiota profiles still significantly differed between C. difficile colonized and non-colonized groups (Monte-Carlo p=0.020, Fig. 2C).

For the 4 infants monitored over time, PCA was realized on TTGE profiles obtained from fecal samples collected between the ages of 2 and 6 months when colonization by C. difficile occurred. Inter-class PCAs with age, subject and C. difficile colonization as instrumental variables showed no significant difference according to age (p=0.128) in this sub-group whereas C. difficile
Bacterial species associated with *C. difficile* colonization status

Statistical analysis of the discriminating variables between infants colonized or non-colonized by *C. difficile* highlighted 3 TTGE bands associated with *C. difficile* colonized infants’ profiles (Cd+ bands) and 4 bands with *C. difficile* non-colonized infants’ profiles (Cd- bands). The same specific bands were identified in the sub-group of infants aged 2 to 5 months. Bands’ position on the gels and samples that were extracted from are shown in Fig. 3. Cd+ bands corresponding 16S rDNA sequences related to *Klebsiella pneumoniae* (band 2), *Ruminococcus gnavus* (band 3) and a relative of *Clostridium nexilae* (band 4). Cd- bands corresponding 16S rDNA sequences corresponded to *Staphylococcus epidermidis* (band 1), *Escherichia coli* (band 5) and *Bifidobacterium longum* subp. *longum* bv. *infantis* (band 6 and 7) as presented in Table 2.

Frequency and intensity of these specific bands were determined on both densitometric curves and gel scans (Fig. 4). All the 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 presented also Cd- bands. Among the 27 *C. difficile* non-colonized infants, 25 had at least one Cd- band and 20 had 2 or 3 Cd- bands (74.7%). Eight of these infants presented also Cd+ bands. Bands intensities’ medians were not significantly different except for *R. gnavus*, which was significantly more abundant in association with *C. difficile*.

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 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 has been well established in babies, 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 FISH combined with flow cytometry and accounted for 0.5 ±1% of the total microbiota (10). 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 (15, 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 inter-individual variability in infant’s 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 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 sub-group of infants aged 2-5 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 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 non-colonized infants’ microbiota. Three bacterial species were identified on both a broad set of infants and during the colonization monitoring in four infants. *Bifidobacterium longum* was associated with fecal microbiota of *C. difficile* non-colonized infants, while colonized infants presented more frequently *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 intra-
individual study. *Staphylococcus epidermidis* and *Escherichia coli* were preferentially found in non-colonized infants and a relative of *Clostridium nexile* in colonized infants. In recent studies in adults, several bacterial groups have been suggested as being associated with protection against *C. difficile* colonization. Hopkins *et al.* analyzed the fecal microbiota in elderly subjects and were able to demonstrate that the bacterial species diversity was markedly lower in CDI patients as compared to healthy controls. In CDI patients, the microbiota was characterized by a high number of facultative anaerobes and low levels of *Bifidobacterium* and *Bacteroides* (13). 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* (10). In our study, the *Bifidobacterium* spp also appears as a characteristic of the non-colonized 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 biopsies (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 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 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 infants group was characterized by higher detection frequency of *Klebsiella pneumoniae*, a facultative anaerobic bacterium naturally resistant to
aminopenicillins. In the work of Hopkins et al., patients with CDI had elevated fecal levels of facultative anaerobes. 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 of the butyrate-producing bacteria such as the *Eubacterium rectale-Clostridium coccoides* group and an increase of the *Bacteroidetes* and *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 the need for the action of antibiotics.

*R. gnavus*, and to a lesser extend a species relative of *C. nexile*, were associated with the presence of *C. difficile*. These two species have been shown to produce a trypsin-dependent antimicrobial substance against *C. perfringens*, but with a lesser activity on *C. difficile* (21). Moreover, *C. nexile* has previously been reported to be stimulated upon antibiotic chemotherapy (7).

In conclusion, our study gives some clues on the microbiota composition allowing *C. difficile* colonization. The presence in important quantity of a *Firmicutes* species such as *R. gnavus* is associated with *C. difficile* colonization. In contrast, presence of *Bifidobacterium* spp appears to be related to 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 on 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 probiotic might help restoring
intestinal microbiota composition.

369 ACKNOWLEDGMENTS

The technical assistance of Karine Le Roux is gratefully acknowledged.
REFERENCES


Table 1. Characteristics of infants and rank tests of Monte-Carlo on inter-class PCAs with clinical and environmental features as instrumental variables of TTGE profiles

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Number of infants per group*</th>
<th>Monte-Carlo Simulated p-value†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>18 (0-2 mo) / 18 (&gt;2-5 mo) / 17 (&gt;5-13 mo)</td>
<td>0.001</td>
</tr>
<tr>
<td>C. difficile culture</td>
<td>27 Cd- / 26 Cd+</td>
<td>0.017</td>
</tr>
<tr>
<td>Feeding</td>
<td>7 Breast / 17 For / 11 Mixed / 11 Div / 3 na</td>
<td>0.022</td>
</tr>
<tr>
<td>Term of birth</td>
<td>8 Preterm / 42 Term / 2 na</td>
<td>0.034</td>
</tr>
<tr>
<td>Season</td>
<td>3 Win08 / 17 Sum08 / 27 Win09 / 6 Sum09</td>
<td>0.150</td>
</tr>
<tr>
<td>Delivery mode</td>
<td>37 Vaginal delivery / 7 Caesarean / 11 na</td>
<td>0.282</td>
</tr>
<tr>
<td>Site</td>
<td>18 Outpatients / 31 Pediatrics / 4 Neonatology</td>
<td>0.527</td>
</tr>
<tr>
<td>TcdA/TcdB</td>
<td>16 A-B- / 10 A+B+</td>
<td>0.584</td>
</tr>
<tr>
<td>Atopy</td>
<td>18 Atopy- / 19 Atopy+ / 16 na</td>
<td>0.721</td>
</tr>
<tr>
<td>Gender</td>
<td>25 F / 28 M</td>
<td>0.978</td>
</tr>
</tbody>
</table>

*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. †Rank test of Monte-Carlo calculate the simulated p-value by extrapolating the test (Hypothesis Ho: significant difference between TTGE profiles of the populations) on 999 replicates. Significant differences are observed between TTGE profiles for p<0.05.
Table 2. Bands specifically associated with *C. difficile* colonization or non-colonization in infants; Characteristics and best sequence isolate hit match using nucleotide BLAST.

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

*Two samples were used for band excision and consecutive DNA extraction on TTGE profile, except for band 4 due to the failure of DNA extraction from one of the two samples.
Figure legends

Figure 1. TTGE fingerprinting profiles of 16S rDNA amplicons (obtained using primers for V6 to V8 regions) of a C. difficile strain and 18 fecal samples from 0-13 months infants (lane 1-18).

Mq: marker consisting of a PCR amplicon mix of 7 cloned rDNAs from different bacterial species. Cd+: positive C. difficile culture; Cd-: negative C. difficile culture.

Figure 2. Principal Component Analysis of TTGE fingerprinting profiles of dominant intestinal bacterial species (fecal samples).


B, C and D: Inter-class PCA of TTGE profiles with instrumental variables. Individuals (represented by black dots or symbols) were clustered (ellipses or bars) and centre of gravity computed for each class.

B: Inter-class PCA of TTGE profiles of 53 infants aged 0-13 months with C. difficile toxigenic culture status as 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: Inter-class PCA of TTGE profiles of 20 infants aged 2-5 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). Age group of the infants was not associated with modifications in the microbiota composition (p=0.125).

D: Inter-class PCA of TTGE profiles of 4 infants aged 2-6 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.

**Figure 3.** TTGE profiles of 16S rDNA amplicons (obtained using primers for V6 to V8 regions) of fecal samples and localization of bands associated with colonization or non-colonization by *C. difficile*.

**Figure 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 unit. ‡Chi square test on frequency. †Mann Whitney U-test (Wilcoxon) on median intensity.
<table>
<thead>
<tr>
<th>Sample</th>
<th>0-3 month</th>
<th>2-4 months</th>
<th>5-10 months</th>
</tr>
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<tbody>
<tr>
<td>Cl</td>
<td>Cd-</td>
<td>Cd+</td>
<td>Cd-</td>
</tr>
<tr>
<td></td>
<td>1</td>
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<tr>
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<td>17</td>
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</tr>
<tr>
<td></td>
<td>Cd- samples (n = 27)</td>
<td></td>
<td>Cd+ samples (n = 26)</td>
</tr>
<tr>
<td>------------------</td>
<td>----------------------</td>
<td>----------</td>
<td>----------------------</td>
</tr>
<tr>
<td></td>
<td>(%) (n)</td>
<td>Median</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>intensity*</td>
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</tr>
<tr>
<td>S. epidermidis</td>
<td>70.4 (19)</td>
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<tr>
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<td>38.0</td>
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<td>K. pneumoniae</td>
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<td>R. gnavus</td>
<td>63.0 (17)</td>
<td>39.0</td>
<td>32.4</td>
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<td>C. Nexile</td>
<td>29.6 (6)</td>
<td>9.5</td>
<td>12.0</td>
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