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Amoxicillin treatment modifies the composition of *Bifidobacterium* species in infant intestinal microbiota

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ABSTRACT

Objectives: Amoxicillin is a beta-lactam antibiotic largely used in childhood. However only few studies described its impact on composition of children gut microbiota, in particular on *Bifidobacterium* populations considered as beneficial microorganisms. In this study, the impact on faecal *Bifidobacterium* species of a seven-day amoxicillin treatment was quantitatively and qualitatively assessed in infants during an episode of acute respiratory infection.
Methods: Faecal samples from 31 infants were obtained on day 0 (just before amoxicillin therapy) and on day 7 (the end of therapy). Total DNA was extracted and bifidobacteria were quantified using real-time PCR. Predominant *Bifidobacterium* species were then identified using specific PCR-TTGE.
Results: Bifidobacteria concentrations were not significantly altered by amoxicillin compared to the healthy group. However, amoxicillin treatment induced a complete disappearance of *Bifidobacterium adolescentis* species (occurrence rate of 0% versus 36.4% in healthy group, $P < 0.001$), a significant decrease in the occurrence rate of *Bifidobacterium bifidum* (23% versus 54.5% in healthy group, $P < 0.05$), but did not affect *Bifidobacterium longum* (93.5% versus 100% in healthy group) and *Bifidobacterium pseudocatenulatum/B. catenulatum* (about 55% in both groups). The number of *Bifidobacterium* species per microbiota significantly decreased from 2.5 ± 1 for healthy group to 1.8 ± 0.9 for treated infants ($P < 0.05$).
Conclusions: This study showed that a 7 day amoxicillin treatment did not alter the counts of *Bifidobacterium*. However amoxicillin can have an impact by changing the microbiota at the species level and decreased the diversity of this population.

1. Introduction

The human colonic microbiota represents an enormous biomass of up to 10^{14} microbial cells composed of several hundred species [1,2]. Although the mature colonic microbiota remains remarkably stable during life [3] it can be disturbed by the administration of antibiotics whether they are administered orally or through injection. Exposure of the colonic microbiota to antibiotics may result in a shift in microbial populations often resulting in gastrointestinal disorders [4–6]. The acute effects of antibiotic treatment on the colonic microbiota range from self-limiting diarrhea to life-threatening pseudomembranous colitis induced by toxigenic *Clostridium difficile* [7].

Among the colonic microbial populations, members of the genus *Bifidobacterium* are the numerically predominant bacteria in the healthy breast-fed infants and are considered as health-promoting microorganisms [8]. However, only few data are available concerning the effects of treatment with antimicrobial agents on these microbial populations.

In vitro susceptibility test showed that the *Bifidobacterium* species isolated from the human colonic microbiota were generally sensitive to penicillin G, amoxicillin, erythromycin, rifampicin, and vancomycin; on the contrary, they are resistant to gentamicin, kanamycin, neomycin and showed variable susceptibility to trimethoprim, ciprofloxacin, tetracycline and metronidazole [9–12]. In children, erythromycin reduced the number of *Bifidobacterium* spp. by around 10 fold and enterobacteria by around 1000 fold during treatment [6]. Gentamicin orally administered caused a 100-fold reduction in the number of enterobacteria and bifidobacteria, reversible after treatment. Administration of penicillin V, ampicillin or methicillin, resulted in a 10^2- to 10^4-fold

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decrease in bifidobacteria in children [6]. Amoxicillin, a beta-lactam antibiotic, inhibitor of bacterial cell wall synthesis, is widely prescribed for the treatment of respiratory tract infections in adults and children. It has been shown that the faecal microbiota of adults displays a major shift in dominant species upon an amoxicillin treatment, starting 24 h after antibiotic initiation and persisting during treatment [13,14]. We have previously shown using fluorescent *in situ* hybridization that a one-week amoxicillin treatment did not alter the total number of bifidobacteria [15]. Fingerprinting methods such as denaturing gel electrophoresis (DGE) based on sequence-specific separation of 16S rRNA gene amplicons, were developed to monitor either bifidobacterial species over time [16–19] or other intestinal phylogenetic groups [20–22]. In a study using *Bifidobacterium*-specific PCR-DGGE (Denaturing Gradient Gel Electrophoresis), profiles of 7 out of 10 children were changed following amoxicillin treatment [23]. However, the identification of the species involved was not detailed in the publication [23].

The aim of the present study was to investigate the quantitative and qualitative changes occurring in the faecal bifidobacterial populations in 18 month-children after a one-week amoxicillin treatment, using real-time PCR (qPCR) and PCR-TTGE (Temporal Temperature Gradient gel Electrophoresis) combined with clone sequence analysis.

2. Material and methods

2.1. Bacterial strains and growth conditions

The reference strains used in this study were purchased in lyophilized form from the Pasteur Institute Collection (CIP, Paris, France): *Bifidobacterium adolescentis* CIP64.59^T, *Bifidobacterium angulatum* CIP104167^T, *Bifidobacterium bifidum* CIP56.7^T, *Bifidobacterium breve* CIP64.69^T, *Bifidobacterium dentium* CIP104176^T, *Bifidobacterium gallicum* CIP103417^T, *Bifidobacterium animalis* subsp. *lactis* CIP105265^T, *Bifidobacterium longum* subsp. *infantis* CIP64.67, *B. longum* subsp. *longum* CIP64.62^T, *B. longum* CIP64.63 and *Bifidobacterium pseudocatenulatum* CIP104168^T. Cells were grown at 37 °C in M20 medium (Pasteur Institute, Paris, France) under anaerobic conditions (Anaerogen™, Oxoid SA, France).

2.2. Clinical data

All infants admitted to the study had to fulfill the following inclusion criteria: age between 12 and 24 months on admission, born at term with a weight between 2.5 and 4.2 kg, with normal bowel function, and on full oral feeding. Furthermore, they must have a diagnosis of acute bronchitis that warranted antibiotic treatment. Acute bronchitis was defined by the presence of productive cough, occasional wheezing and the absence of overt bronchial obstruction, lung consolidation, or pleural involvement. Children were excluded if they fulfilled one or more of the following criteria: antibiotic use in the preceding 4 weeks, allergy to antibiotics, suspected to require antibiotic treatment >7 days, total or partial breastfeeding, and treatment with steroids or prokinetic drugs. The antibiotic treatment was started in the Health Center "La Faena" located in the Southeastern part of Santiago, Chile, on the day of protocol admission and consisted of an amoxicillin suspension (Amoval, Laboratorios Saval, Chile) at a dosage of 50 mg/kg/d, divided into three daily doses and administered for 7 days. Faecal samples from 31 infants were obtained on day 0 (just before antibiotic therapy) and on day 7 (the end of therapy). Two faecal samples from 11 healthy infants who had not received antibiotics within the previous months were collected with a 7-day interval. Stools were collected immediately after defecation and were transported in a cooler at 4 °C to the laboratory where they were stored at −30 °C for less than 2 months. All parents gave their informed consent for this study, according to the protocol approved by the Committee on Ethics in Research on Humans, INTA, University of Chile.

2.3. DNA extraction

Stools were thawed, homogenized and 125 mg aliquot was used to extract total DNA using the chemical guanidium isothiocyanate and the mechanical bead beating method as previously described [18,24].

2.4. Real-time PCR for Bifidobacterium and total bacterial quantification

Reactions were performed in duplicate in a volume of 25 μl within 96-well twin-tech PCR plates, using the Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen, Cergy Pontoise, France). The forward and reverse primers used were Bif164f, Bif662r for *Bifidobacterium* genus [25] and Bia339f, Bia788r for total bacteria [17]. Amplifications were performed in a Mastercycler ep Realplex4 (excitation source 470 nm, emission 520/550 nm) (Eppendorf AG, Hamburg, Germany) with the following temperature profile: one cycle at 96 °C (2 min), 40 cycles of denaturation at 96 °C (15 s), primer annealing at 62 °C (1 min) for bifidobacteria and at 55 °C (1 min) for total bacteria, and elongation step at 68 °C (2 min). Finally, the melting curve was made by slowly heating the PCR mixtures from 60 to 96 °C (20 min) with simultaneous measurements of the SYBR Green I signal intensities. A standard curve made from known amounts of plasmid DNA containing a 16S rRNA gene insert from *Bifidobacterium* or *Escherichia coli* allowed quantifications.

2.5. PCR for bifidobacterial TTGE; sequences analyses

The primers Bif164f and Bif662r [25] were used to amplify the 16S rRNA genes of the *Bifidobacterium* genus from samples. PCR products were separated on TTGE, using a Dcode™ system (Bio-Rad laboratories, Hercules, CA, USA) and analysed as previously described [18]. The relative front (Rf) was calculated for each band. This parameter is defined as the distance from the top of a defined lane from gel to the band. One standard consisting of a mixture of PCR products obtained from identified species was run alongside the faecal samples (first, middle and last lanes). In order to compare different gels and identify the *Bifidobacterium* species present in the faeces, normalization of Rfs for the bands within the standard was performed. Twenty-six bands were excised from gels, cloned into pGEM®-T (Promega Corporation, Madison, WI, USA) and subsequently sequenced using primer Bif164f (Cogenics-Genome Express, Meylan, France). The selection criterion to excise bands was their absence in our Rf data bank. Some bands with Rf present in the bank were also sequenced to confirm the identification. The sequences were corrected using Chromas version 1.45. A search of the GenBank nucleotide database was conducted using the BLAST algorithm to determine the closest relative of partial 16S rRNA gene sequences. Partial representative sequences were deposited in GenBank (accession numbers FJ617455 to FJ617475). The last 5 bands cloned corresponded to heteroduplexes.

2.6. Data analysis

Quantitative data were presented as $\log_{10}$ 16S rRNA gene copy number per g of wet weight of faeces. All other results are expressed as means ± standard deviation (SD). Statistical analyses were performed with Statgraphics plus 5.1 package (Statistical Graphics Corp., Rockville, MD, USA) using the Fisher's exact test for

categorical data (species percentages) and the Wilcoxon *T* test for quantitative data.

3. Results

3.1. Infants

Table 1 presents the characteristics of the children. The 42 subjects enrolled in the study protocol had a normal clinical examination except for the diagnosis of acute bronchitis for 31 subjects treated with amoxicillin. The 31 subjects successfully completed the 7 day antibiotic treatment.

3.2. Quantification of total bacteria and bifidobacteria in faecal samples

Total 16S rRNA gene copy numbers at day 0 was 9.7 ± 0.5 $\log_{10}$ for healthy group and 10 ± 1 $\log_{10}$ for treated group. The same results were observed for the two groups at day 7. Faecal samples had bifidobacterial 16S rRNA gene copy numbers ranging from 5.2 to 10.9 $\log_{10}$. Only one infant from healthy group had very low counts of bifidobacteria (5.2 $\log_{10}$ 16S rRNA gene copies) versus two infants in treated group (6.5 $\log_{10}$ 16S rRNA gene copies and 6.8 $\log_{10}$ 16S rRNA gene copies). Mean 16S rRNA gene copy numbers of *Bifidobacterium* in healthy infants were 9.1 ± 1.4 $\log_{10}$ at day 0 and 9.2 ± 0.5 $\log_{10}$ at day 7 while in treated infants they were 9.3 ± 1 $\log_{10}$ at day 0 and 9.6 ± 0.9 $\log_{10}$ at day 7. Statistical analyses did not show any differences between healthy and treated infants at day 0 or at day 7 concerning total bacteria or bifidobacteria. Moreover, intragroup statistical analyses did not show any significant differences between d0 and d7 for total bacteria or bifidobacteria.

3.3. Impact of antibiotic therapy on bifidobacterial microbiota

The TTGE profiles were composed of one to seven bands, as observed in Fig. 1. Most fragments co-migrated to the same position than the reference strains but a few migrated to a different position and could not be identified in this way. Band sequencing (Genbank accession numbers FJ617455 to FJ617475) resulted in the characterization of *B. longum* (TTGE band normalised relative front (Rf) 0.56; 0.57; 0.61; 0.62; 0.63), *B. bifidum* (Rf 0.45; 0.48), *B. breve* (Rf 0.55), *B. adolescentis* (Rf 0.35; 0.37; 0.41; 0.58; 0.69; 0.72) or *B. pseudocatenulatum/B. catenulatum* (Rf 0.59; 0.68; 0.74; 0.75).

Results show that at day 0, there were no significant differences in the occurrence of any of the *Bifidobacterium* species between healthy and treated infants. The most common bands corresponded to Rf of *B. longum* and were seen in 90.5% of the TTGE profiles (90.3% in treated infants versus 90.9% in healthy group; NS). Bands migrating to *B. pseudocatenulatum/B. catenulatum* group Rf were detected in 57.1% of the TTGE profiles (64.5% in treated infants versus 36.4% in healthy group; NS). Then there were the *B. bifidum* bands found in 47.6% of the TTGE profiles (48.4% in treated infants versus 45.4% in healthy group; NS). Finally *B. adolescentis* bands were ranged from 16.1% in the treated group samples up to 27% in the healthy group samples (NS). A *B. breve* band was detected in only one TTGE profile and another migrating as *B. dentium* was detected in two profiles. No band corresponding to Rf of *B. angulatum* was detected from any of the subjects.

The occurrence rates of the *Bifidobacterium* species observed at day 7 for healthy and treated infants are shown in Fig. 2. Amoxicillin treatment did not affect *B. longum* (at least one *B. longum* band was seen in 93.5% of the TTGE profiles versus 100% in healthy group samples) or *B. pseudocatenulatum/B. catenulatum* (at least one band in about 55% of the TTGE profiles in both group samples) but induced the complete disappearance of *B. adolescentis* (0% versus 36.4% in healthy group samples, $P < 0.001$) and a significant decrease of *B. bifidum* occurrence (23% versus 54.5% in healthy group samples, $P < 0.05$).

At the intragroup level, the TTGE bands corresponding to Rf of *B. adolescentis* disappeared at day 7 in TTGE profiles of 5 treated children among 5. The TTGE band corresponding to *B. bifidum* disappeared in TTGE profiles of 9 treated children among 15 and appeared only once in TTGE profiles of the 16 other treated infants. Bands from *B. pseudocatenulatum/B. catenulatum* group disappeared in 5 TTGE profiles among 20 and appeared twice in TTGE profiles of the 11 other treated infants. The TTGE bands corresponding to *B. longum* did not change in 27 TTGE profiles among 28, disappeared in one and appeared in two new profiles.

At day 0, the average number of *Bifidobacterium* species per microbiota for healthy group was not significantly different from treated infants (2.1 ± 0.9 *vs* 2.2 ± 1). At day 7, the average number of *Bifidobacterium* species per microbiota was significantly lower for treated infants compared to healthy group (1.8 ± 0.9 *vs* 2.5 ± 1) ($P < 0.05$).

Table 1
Characteristics of infants (mean [range]).

	Total	Healthy group	Treated group
	$n = 42$	$n = 11$	$n = 31$
Gender	23/19 M/F	5/6 M/F	18/13 M/F
Age (m)	18.2 [12–23]	18.7 [15–23]	18 [12–23]
Weight (kg)	10.9 [9.1–13.6]	10.7 [9.2–12.3]	11 [9.1–13.6]
Size (cm)	82 [75–90]	81 [76–85]	83 [75–90]

4. Discussion

In this study, the impact of a seven-day amoxicillin treatment on faecal *Bifidobacterium* species was quantitatively and qualitatively assessed in 18 month-infants suffering from an episode of acute respiratory infection compared with a healthy group. The counts of total bifidobacteria at day 7 were not significantly altered by amoxicillin. No significant difference was observed at day 7 between healthy and treated infants for total bacterial counts. However, data were presented as $\log_{10}$ 16S rRNA gene copy numbers, consequently species changes such as an increase of enterobacteria described in a previous study [15] could have been masked by this global estimation.

Numerous publications reported on the modification of faecal microbiota but only at genus level. TTGE was performed to monitor the diversity of *Bifidobacterium* species before and at the end of treatment at the species level. One to seven bands were observed in each profile as already reported [17]. Cloning and sequencing were performed to identify 26 bands of interest. The same identifications were obtained for bands with identical Rf. Due to the presence of several ribosomal operons (2–5 copies) in human *Bifidobacterium* strains [26] with different sequences, the number of bands do not systematically reflect the number of species. For example, the type strain of *B. adolescentis* species displayed 3 TTGE bands, the first corresponding to 4 copies of the ribosomal operon, the second to the fifth copy and the third in the upper part of the gel being a heteroduplex due to the presence of the two others during the PCR [19]. The sequencing allowed the identification of 3 new bands for *B. longum*, one new band for *B. bifidum*, 3 new bands for *B. adolescentis*, and 3 other bands for *B. pseudocatenulatum/B. catenulatum*. The PCR-TTGE method allowed the differentiation of bands with very close Rf. As previously shown for the *B. adolescentis* type strain [19], heteroduplexes were identified in the upper part of the gel (bands 6, 7 and 9; Fig. 1). For example, after cloning of bands 7, PCR from colonies only produced either band 4 or band 8 present in the previous TTGE profile but none corresponded to upper

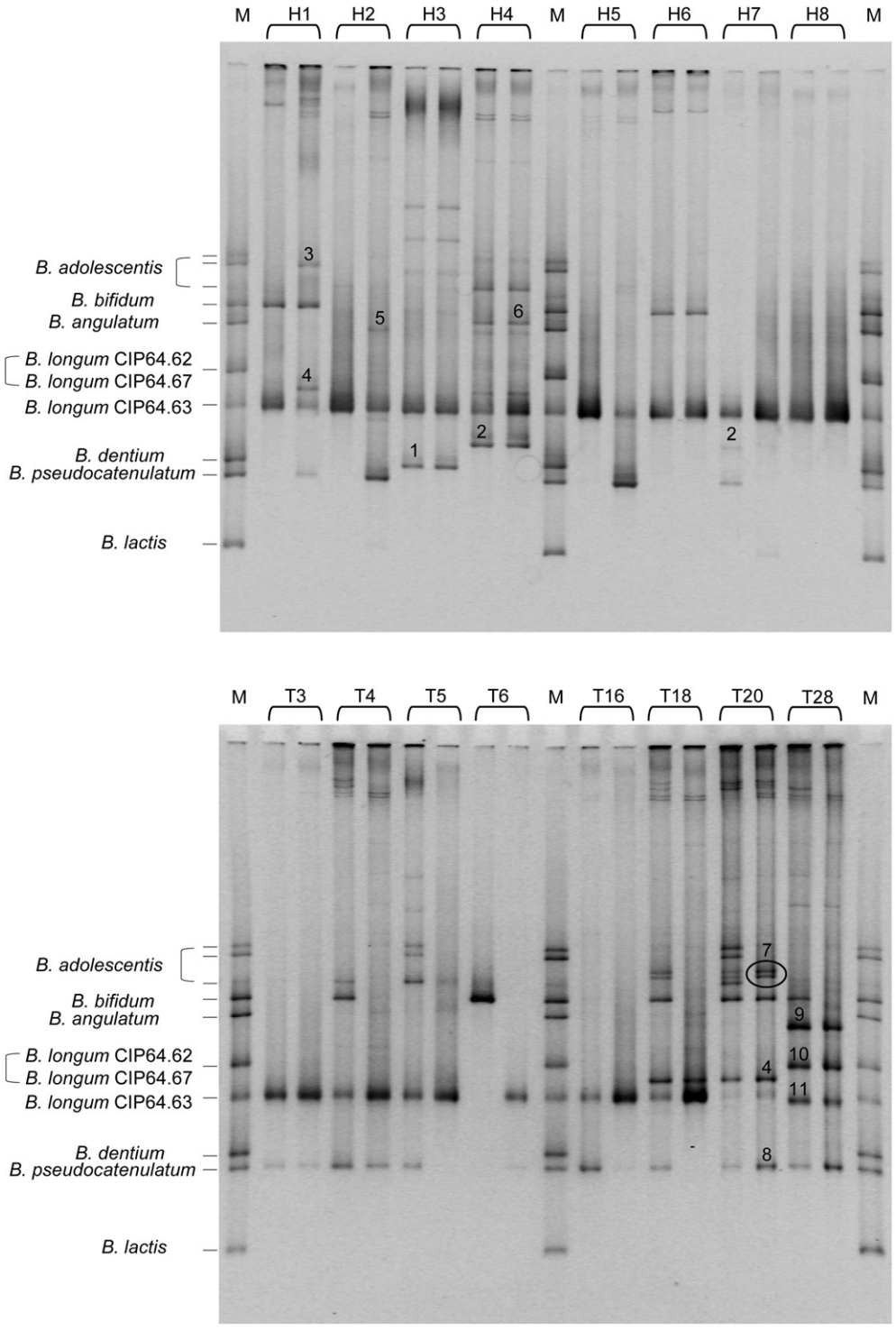

Fig. 1. TTGE profiles representing the bifidobacterial diversity of faecal samples from healthy (C) and amoxicillin-treated (T) microbiota. For each infant, first profile corresponds to day 0 and second to day 7 samples. 1: *B. adolescentis*; 2:*B. pseudocatenulatum/B. catenulatum*; 3:*B. adolescentis;* 4: *B. pseudocatenulatum/B. catenulatum*; 5:*B. bifidum*; 6: heteroduplex; 7: heteroduplexes created by the presence of bands 4 and 8; 8: *B. pseudocatenulatum/B. catenulatum*; 9: heteroduplex created by the presence of bands 10 and 11; 10: *B. longum*; 11: *B. longum.*

fragments 7. A mix of fragments 4 (*B. pseudocatenulatum*) and 8 (*B. pseudocatenulatum*) was amplified using PCR and produced the same four TTGE bands (data not shown). The same occurred for the other heteroduplexes.

One to four *Bifidobacterium* species were identified in each microbiota, regardless the group of infants. However, the mean species number is significantly lower at day 7 (1.8 ± 0.9) for treated infants compared to healthy group (2.5 ± 1) ($P < 0.05$). Similarly, a marked decrease in the diversity of *Bifidobacterium* populations was observed during doxycycline therapy: the average number of amplicons detected by PCR-DGGE was 0.8 in the antibiotic group versus 4.3 in the control group [27].

Monitoring of *Bifidobacterium* species showed no significant difference in species occurrence at day 0 between both groups.

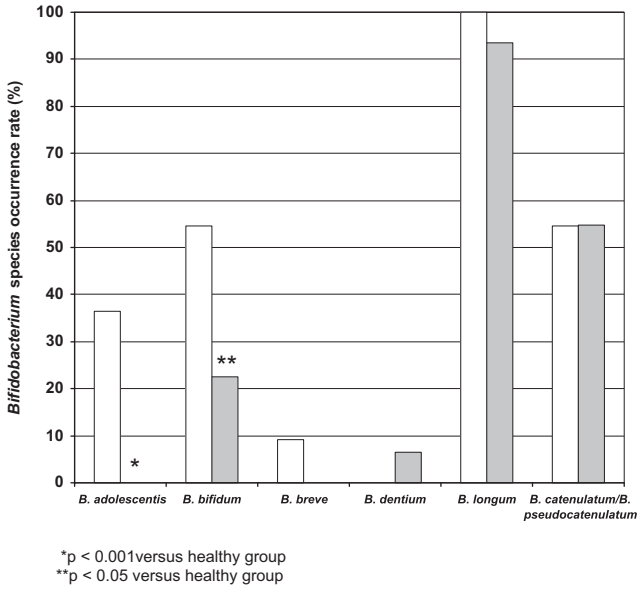

Fig. 2. Occurrence rate of *Bifidobacterium* species observed at day 7 for healthy (□) and amoxicillin-treated (▩) microbiota.

These occurrences were close to those described in a previous study using the PCR approach [28] for 30 clinically healthy one year-old children [29]. However, at day 7, significant alterations for some species were observed. *B. adolescentis* disappeared in treated group (0% versus 36% in healthy group, $P < 0.001$) and *B. bifidum* decreased significantly (23% versus 55% in healthy group, $P < 0.05$). In some cases, other species not present at day 0 and probably belonging to the subdominant microbiota, appeared at day 7, like *B. longum* or *B. pseudocatenulatum*/*B. catenulatum* (Fig. 1; T6). The occurrence of these two last species remained stable between the two groups. As explained in previous studies, the antimicrobial effect is dose-dependent and amoxicillin showed variable MIC (minimum inhibitory concentration) depending on species or strains tested [9,12]. No resistant *Bifidobacterium* strains were detected *in vitro* with 25 μg/mL of amoxicillin [12]. Generally, *B. adolescentis* and *B. bifidum* seemed to be more susceptible *in vitro* (MIC range ≤0.06–0.5 mg/L) than was *B. longum* (MIC range ≤0.06–2 mg/L) [9,12]. Thus, our results could be explained by MIC. Noticeably, the relative intensity of bands was often increased for *B. longum* at day 7 compared to day 0 (Fig. 1; T4, T5, T16 and T18) indicating a higher relative quantity of this species. At day 7, the TTGE band corresponding to Rf of *B. bifidum* (MIC range ≤ 0.06–0.5 mg/L) disappeared in TTGE profiles of 9 treated children among 15. Eleven *B. pseudocatenulatum* strains were tested *in vitro* and had MIC values close to *B. bifidum* (MIC range ≤0.06–0.5 mg/L) [9,12]. Mean occurrence rates of *B. pseudocatenulatum/B. catenulatum* did not reflect internal variations since bands from this species disappeared in 5 TTGE profiles among 20 and appeared twice in TTGE profiles of the 11 other treated infants. In our study, *B. breve* was found in microbiota from only one healthy infant at day 0 and day 7. This species was previously detected in dominance in babies [17] and had an occurrence rate of 20.7% within 30 one year-old tested children but was not found in eight 6-year-old children [29].

In healthy microbiota, variations were also observed between each pair of TTGE profiles. Mean similarity percentage was 73% for healthy infants, ranging from 50% to 100%, versus 65% for treated infants ranging from 0% to 100%. Monthly monitoring of *Bifidobacterium* species in 6 adults during 8 months showed high variations in the population and composition for some ones and very stable bifidobacterial microbiota for others [30]. Molecular monitoring of intestinal *Bifidobacterium* strains in 4 adults using ribotyping showed no significant changes 30 days and 90 days after an amoxicillin antibiotherapy [31]. Each microbiota remained stable in terms of strain and species composition over time, with slight variations, also detected in the control subject [31].

A shift in *Bifidobacterium* species could have a physiological impact on the host. For example, it has been recently shown that *Bifidobacterium*'s capacity to stimulate immunity is species specific but its influence on the orientation of the immune system (T_H1, T_H2 cytokines, no effect) operates in a strain specific manner [32–34]. Ouwehand and colleagues have reported that the prevalence of *B. adolescentis* differed in the faeces of healthy and allergic Finish children aged 2–7 months. Six out of seven allergic children harboured *B. adolescentis* in the faeces, whereas this species was not detected in six healthy children [35]. The same was observed in 5-year-old allergic children [36]. Only some strains of *B. longum* subsp. *longum*/*infantis* can protect against the lethal infection of *E. coli* O157-H7 by preventing Shiga toxin production in the caecum and/or Shiga toxin transfer from the intestinal lumen to the bloodstream [37]. Moreover, by changing the intestinal microbiota balance, antibiotic treatment may lead to a homeostatic imbalance through alterations in expression of IEC tight junction proteins, mucin, antimicrobial peptides, and cytokines [38]. However, some resilience of the microbiota can exist. A previous study demonstrated the resilience of the dominant faecal microbiota from 5 out of 6 adults, 30 days following amoxicillin treatment with average similarities to day 0 of 88% for 5 subjects and below 70% for the last one [13]. Resilience was also obtained for three individuals 30 days after a 5-day ciprofloxacin treatment, with a community composition closely resembling its pretreatment state, but several taxa failed to recover within 6 months [39].

5. Conclusions

In conclusion, this study showed that in children, a 7 day amoxicillin treatment did not significantly alter the mean genus *Bifidobacterium* 16S rRNA gene copy faecal concentration. However this treatment can have an implication on *Bifidobacterium* species composition depending on the species present before treatment. Moreover it significantly decreased the diversity of this population.

As the bifidobacterial populations are considered to be important for a well-balanced intestinal microbiota, the possible consequences of a shift in *Bifidobacterium* species on the physiology of the host remained to be assessed.

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