

Clinical Microbiology

Diversity of gut *Bifidobacterium* species is not altered between allergic and non-allergic French infantsA.J. Waligora-Dupriet^{a,*}, F. Campeotto^{a,b,1}, K. Romero^a, I. Mangin^e, G. Rouzaud^a, O. Ménard^a, A. Suau^e, P. Soulaïnes^{b,1}, I. Nicolis^c, N. Kapel^{a,d}, C. Dupont^{b,1}, M.J. Butel^a^a EA 4065, Ecosystème Intestinal, Probiotiques, Antibiotiques, Faculté des Sciences Pharmaceutiques et biologiques, Université Paris Descartes, 4 avenue de l'observatoire, 75006 Paris, France^b Néonatalogie et Gastroentérologie Pédiatrique, APHP, Groupe Hospitalier Cochin-Saint Vincent de Paul, Université Paris Descartes, France^c EA 4466 et Département de Santé Publique et Biostatistiques, Faculté des Sciences Pharmaceutiques et Biologiques, Université Paris Descartes, France^d Coprologie Fonctionnelle, APHP, Groupe Hospitalier Pitié-Salpêtrière, Paris, France^e Laboratoire de Biologie EA 3199, Conservatoire National des Arts et Métiers, Paris, France

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ABSTRACT

Some clinical studies have suggested a relationship between allergic diseases and gut microbiota. We aimed to study bifidobacterial colonization at species and strain levels in ten allergic French infants included at their first clinical consultation and 20 controls matching for age at sampling, mode of delivery, *per partum* antibiotics, type of feeding and antibiotics in the first weeks of life. The faecal microbiota was analyzed by culture methods and TTGE. *Bifidobacterium* species and strains were identified using multiplex PCR and Box-PCR fingerprinting.

No differences were observed between groups in the number of colonized infants or in the levels of colonization by the main aerobic and anaerobic genera. All infants were colonized with high levels of *Bifidobacterium* except for one in each group. One to 5 *Bifidobacterium* species and 1 to 7 strains were observed per subject independently of allergic status and age at sampling. Our study showed the infants to be colonized by several species and strains, including several strains from the same species. This diversity in *Bifidobacterium* colonization was not related with the allergic status and showed that the link between *Bifidobacterium* colonization and allergic diseases is complex and cannot be restricted to the role attributed to *Bifidobacterium* species.

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1. Introduction

Intestinal commensal bacteria play a crucial role in immune response through the maturation of the intestinal immune system and through the modulation of the Th₁/Th₂ balance [1,2]. Differences in the composition of the bacterial community in the faeces of children who live in countries with high or low prevalence of allergy, as well of children with or without allergic diseases have been reviewed by Penders et al. [3]. In particular, several authors have pointed out some quantitative and qualitative differences in colonization with *Bifidobacterium*, a dominant genus in infant faecal microbiota which may provide beneficial effects [4]. Indeed,

a decrease in bifidobacterial levels has been associated with allergic status [5–8] although these results have been contradicted [9–11]. Differences in species have also been observed but no clear consensus exists: *B. adolescentis* has been associated with the faecal microbiota of both allergic infants [12,13] and non-allergic infants [14]. Similarly, *B. catenulatum/pseudocatenulatum* has been associated with both allergic infants [15] and non-allergic ones [16]. Moreover, some others have reported a reduction of *Bifidobacterium* diversity to be linked with allergy [16] but once again, this was not confirmed in another study [14]. All these studies associated modifications in prevalence and species composition of bifidobacteria to the development of the allergic phenotype based on the hypothesis of early immune stimulation by intestinal microbiota. However, *in vitro* and *in vivo* studies have shown that immunostimulatory properties of *Bifidobacterium* are strain-specific [17,18].

In this work, we aimed to study faecal microbiota focusing on the diversity of bifidobacterial colonization in allergic and non-allergic French infants at species and strain levels. To avoid any

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alteration in the microbiota due to the care of allergic disease, sampling was performed at the time of diagnosis, i.e. before any food exclusion and/or treatment. Moreover, faecal calprotectin was measured as a marker of intestinal inflammation.

2. Subjects and methods

2.1. Study population and sample

Twenty infants were included at St Vincent de Paul hospital in Paris at their first clinical consultation in gastroenterology department for suspicion of allergy, i.e. before any food exclusion and/or treatment. For each patient with allergic symptoms i.e. atopic eczema/dermatitis syndrome (AEDS), wheezing and/or digestive symptoms, two controls were matched for their age at sampling and three of the four following clinical parameters: mode of delivery, *per partum* antibiotherapy, type of feeding and antibiotic treatment in the first weeks of life. Allergy was diagnosed with at least one positive prick test and/or atopic patch test and/or radioallergosorbent test (RAST). Children were excluded from the study if they had an acute illness or had taken any treatment, including antibiotics, within 14 days before stool collection.

Two faecal samples were collected at inclusion in sterile tubes. The tube for microbiota analyses contained 0.5 ml brain heart infusion broth with 15% glycerol as a cryoprotective agent. All samples were immediately stored at -80°C until analyses.

This study was approved by the human subjects review committee (CPPRB) of Ile de France (France) and written parental consent was obtained for each inclusion.

2.2. Measurement of faecal calprotectin

Faecal calprotectin concentration was determined using a commercial ELISA (Calprest, Eurospital, Italy) according to the instructions of the manufacturer.

2.3. Analysis of the faecal microbiota

Qualitative and quantitative analysis of faecal microbiota was performed as previously described [19]. Briefly, serial dilutions of stools (10^{-2} to 10^{-6}) in a pre-reduced peptone liquid medium were spread on various media allowing the isolation and quantification of the main genera. Tryptcase soy agar (BioMérieux, Marcy l'Etoile, France) was used to detect all aerobes; Drigalski agar (BioRad, Marnes la Coquette, France) for enterobacteria; D coccosel agar (BioMérieux, Marcy l'Etoile, France) for enterococci, Chapman 110 agar (VWR, Strasbourg, France) for staphylococci. For anaerobic bacteria, the dilutions were plated on cysteine (160 mg/l) Columbia agar base supplemented with sheep blood 5% and neomycin 100 mg/l for total anaerobes, kanamycin 7.5 mg/l and vancomycin 100 mg/l for *Bacteroides*; whole milk 5%, colistin 10 mg/l and neutral red 40 mg/l for clostridia, and CCFA agar (BioMérieux, Marcy-l'Etoile, France) for *Clostridium difficile*. For bifidobacteria, Wilkins Chalgren agar base containing D-glucose 10 g/l, kanamycin 7.5 mg/l, L-cysteine 0.5 g/l and Tween 80 (0.5% v/v) was used [20]. Aerobic cultures were examined after 24 h of incubation and anaerobic ones after 48 h and again after 5 days of incubation in an anaerobic cabinet (MACS, Chemunex AES Laboratoire, Bruz, France) under $\text{H}_2/\text{CO}_2/\text{N}_2$ (10/10/80). Bacterial counts were expressed as the $\log_{10}$ CFU/g of faeces with a count threshold of 3 $\log_{10}$ CFU/g of faeces. Routine laboratory methods and Wadsworth laboratory procedures [21] allowed identification of aerobic and anaerobic bacteria, respectively.

2.4. Identification of bifidobacteria at the genus and species levels

On isolates, identification of bifidobacteria was performed as previously described [24] according to the gram, the morphologic characteristics and PCR amplification with 16S rDNA-gene targeted genus-specific primers adapted from Kok et al. [23] using chromosomal DNA from cultured *Bifidobacterium* isolates, extracted with Puregene genomic DNA isolation kit (Gentra) according to the instructions of the supplier. Species identification was performed using multiplex PCR with 16S rDNA-gene targeted species specific primers adapted from Mullié et al. [25] and 16S rDNA sequencing (Beckman Coulter Genomics SA, Grenoble, France).

On stools, using PCR coupled with temporal temperature gel electrophoresis. Stools were thawed, homogenized and 125 mg aliquot protected by guanidium isothiocyanate was used to extract total DNA using the mechanical bead beating method as previously described [22]. The primers Bif164f and Bif662r [23] were used to amplify the 16S rRNA genes of the *Bifidobacterium* genus from samples as previously described [22]. PCR products were separated using TTGE, with a Dcode_ system (Bio-Rad laboratories, Hercules, CA, USA) and analyzed as previously described [22]. 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. Two standards consisting of a mixture of PCR products obtained from identified species or clones were run alongside the faecal samples (M1 and M2). In order to compare different gels and identify the *Bifidobacterium* species present in the faeces, normalization of Rfs for the bands within the standards was performed. Identifications were based on comparative analysis of the distance migration with many cultured collection bifidobacteria and many clones present in our data bank.

2.5. BOX-PCR fingerprinting

PCR-fingerprinting with the repetitive sequence-based oligonucleotide primer BOXA1R (5'-CTACGGCAAGGCGACGTGACG-3') was adapted from Masco et al. [26]. Each 25 μl PCR reaction contains 2.5 U of taq polymerase (Invitrogen), 1.25 mM of each dNTPs, 8 mM of MgCl_2 , 10% DMSO, 2 μM of primer, and 50 ng of DNA. Amplification was performed as follows: an initial denaturation step (7 min at 97°C) was followed by 30 cycles (denaturation for 1 min at 94°C , hybridization for 1 min at 50°C and elongation for 8 min at 72°C) and a final extension at 72°C for 10 min. The amplified fragments were visualized on agarose 1.6% gel after a 16 h migration at a constant voltage of 55 V. Samples were analyzed using Dice's correlation coefficient and UPGMA method. Isolates with >90% similarity were considered identical [27].

2.6. Statistical analysis

Qualitative variables, i.e. infants' characteristics and number of colonized infants, are reported as frequencies and percentages. Bacterial colonization levels are reported as the median and range. Comparisons between the groups were performed using Fisher's exact test for qualitative parameters, and Man and Whitney test for continuous variables. Logistic regression was used to evaluate bifidobacterial diversity between groups. Spearman's rank correlation coefficient was calculated to investigate whether calprotectin correlated with age, and bifidobacterial colonization. *p*-values <0.05 were considered significant.

3. Results and discussion

To study the link between bifidobacterial diversity and allergy, we focused on infants less than two years old when gut microbiota

Table 1
Clinical data of allergic infants.

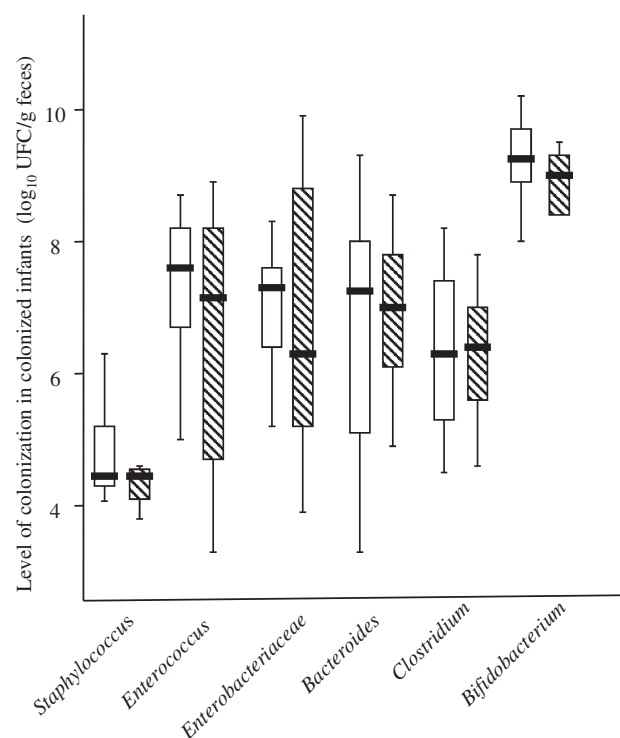
Infant number	Infants characteristics					Positive diagnostic tests			Allergens				
	Age at sampling (months)	Caesarian section	Birth term > 39 SA	Breast-feeding	per partum antibiotherapy	Specific IgE	Prick test	Atopic patches test	Cow milk	Egg	Mustard	Dust-mit	Wheat
1	24		●			●		●	●				
2	7	●	●	●	●	●	●		●	●			
3	3		●	●				●	●				
4	7		●	●			●		●				
5	11		●			●	●	●		●	●	●	●
6	7		●		●			●	●				
7	7			●		●	●		●	●		●	
8	12	●		●				●	●				
9	6			●	●	●	●		●	●			
10	11	●	●			●	●		●				

is not yet fully established. Indeed, many studies emphasize that the time of exposure to environmental factors may be essential to promote beneficial or harmful effects regarding the development of atopic diseases [3]. The allergic infants were infants selected at the time of the diagnosis, before food eviction or any treatment for allergy that could modify the microbiota and the immune environment of the mucosa. Allergy diagnosis was confirmed in 10 infants out of 20, aged between 3 and 24 months old. Two of them had digestive symptoms, 7 had AEDS, one had AEDS associated with wheezing, and all of them were sensitized against a food allergen (Table 1). Controls were matched on the factors which might influence the establishment of the gut microbiota. In the 30 infants studied, 28 (93%) were vaginally delivered, 9 (28%) were born from mother treated by *per partum* antibiotics, 20 (67%) were breastfed and 9 (28%) received antibiotics in the first weeks of life.

Faecal calprotectin, a non invasive evaluation of intestinal inflammatory status, exhibited values remaining in the reference ones for this age. Although the median was two-fold higher in the allergic group (100 µg/g, range <15–412 µg/g) than in controls (52 µg/g, range <15–255 µg/g), no significant differences between the two groups were observed. Faecal calprotectin correlated negatively with age ($\rho = -0.550$, $p = 0.018$) as described by Rugtveit & Fagerhol [28] but only in controls, not in the allergic group. This might reflect modifications of the inflammatory status in allergic children, in agreement with the increase of intestinal permeability in allergic diseases [29].

The faecal microbiota of the 10 allergic and 20 control infants was analyzed in terms of the number of colonized children and the mean level of colonization for each bacterial group (Table 2 and Fig. 1), showing no differences for the main aerobic and anaerobic genera between groups. Concerning the facultative aerobic microbiota, nearly all infants (over 80%) were colonized at high levels with enterococci and enterobacteria (median, respectively, 7.6 and 7.3 log₁₀ CFU/g of faeces) whereas fewer infants were colonized with *Staphylococcus* sp. (80% and 53% respectively in allergic and

control infants) and at low level (median 4.6 log₁₀ CFU/g of faeces) for both. Concerning the anaerobic microbiota, *Bacteroides* and *Clostridium* exhibited high levels for 90% of allergic infants and respectively for 63% and 79% of control ones (Fig. 1). *C. difficile* was found in 60% of allergic infants and 42% of control ones. Such high rates of asymptomatic carriage of *C. difficile* had already been reported in Europe in the faeces of healthy infants [30–32]. The other species of *Clostridium* isolated were mainly *C. perfringens*, *C. butyricum*, *C. paraputrificum* and *C. ramosum*. *Bifidobacterium* (median 9.2 log₁₀ CFU/g of faeces) was observed at high levels in all infants except one in each group. Lactobacilli were seldom detected with a median level of 5.0 log₁₀ CFU/g of faeces in only 4 infants belonging to the two groups. The lack of differences between allergic and control infants is in accordance with the European multicentric prospective study of Adlerberth et al. [33] in which the microbiota was studied using a culture method.

**Fig. 1.** Level of colonization with main facultative anaerobic and strict anaerobic genera in colonized infants represented as a box plot. The box plot shows median (central horizontal line), the 25th centile (lower box border), and the 75th centile (upper box border). The lower and upper horizontal lines refer to the 10th and the 90th centile, respectively. No significant differences were observed between the two groups.**Table 2**
Number (percentage) of colonized infants in the allergic and control groups. No significant differences were observed between the 2 groups.

	Allergic group (n = 10)	Control group (n = 20)
<i>Aerobes</i>		
Staphylococci	8 (80%)	11 (55%)
Enterococci	10 (100%)	17 (85%)
Enterobacteria	10 (100%)	19 (95%)
<i>Anaerobes</i>		
<i>Bacteroides</i>	9 (90%)	12 (60%)
<i>Clostridium</i>	9 (90%)	16 (80%)
<i>Bifidobacterium</i>	9 (90%)	19 (95%)

Table 3
Bifidobacterium species and strains in 10 allergic and 12 control infants: ●, detected using culture method; Δ, detected using TTGE; NA, not applicable.

Infant number	Allergy Yes/No	Bifidobacterium sp.						Culture method		TTGE	
		bifidum	longum		breve	catenulatum group	adolescentis	dentium	Number of species	Number of Box-PCR profiles i.e. strains	Number of species
			subsp longum	subsp infantis							
1	Yes	●△	●△		●	●△			4	4	3
2	Yes		●△		●△				2	2	2
3	Yes	●△	●△		●				3	3	2
4	Yes		●△		△				1	1	2
5	Yes				●△	△		△	1	2	3
6	Yes		△		●△				1		2
7	Yes		●△		●	●△		●△	5	7	3
8	Yes	△	●△	●	●	●		△	4	5	3
9	Yes	●△	●△		●△			●△	4	5	4
10	Yes	△	△			△		△	NA		4
11	No	●△	●△		●				3	3	2
12	No		●△		●△				2	4	2
13	No				●	●△			2	2	1
14	No		●△					●△	2	4	2
15	No		●△		●	●△			3	3	2
16	No	●△			●△				2	3	2
17	No							●	1	1	NA
18	No	●			●				2	2	NA
19	No		△		●△			△	1	1	3
20	No				●△			●△	2	3	2
21	No	●△			●	●△			3	4	2
22	No				△				NA	NA	1

We analyzed bifidobacterial colonization at species level in 20 infants (the 9 colonized in the allergy group and 11 matched controls). *Bifidobacterium* isolation was performed on a media developed in our laboratory which was shown to be efficient for selection of *Bifidobacterium* of human origin and to have a sensitivity equivalent to molecular methods [20,24]. A median number of 12 colonies of *Bifidobacterium* were picked out per infant (range 8–17) for identification. Considering that infants could be colonized by one to three equally distributed dominant species of *Bifidobacterium*, multinomial law probabilities yield a 0.77% risk of missing one of the three species when picking 12 colonies.

The most frequently detected bifidobacteria was *B. breve* (Table 3), occurring in 80% of infants independently of allergy status,

being also the most often detected species in other studies in healthy infants [34–36]. These studies also demonstrated the second place by frequency order of the *B. longum* group similarly to our study: *B. longum* subsp. *longum* was isolated in 78% of allergic infants but only in 36% of controls. Only one isolate of *B. longum* subsp. *infantis* was detected. *B. bifidum*, *B. pseudocatenulatum* and *B. adolescentis* colonized respectively 35%, 30% and 20% of the infants, independently of allergy status. *B. dentium* was rare and *B. angulatum* and *B. animalis* absent. Using TTGE, similar results were obtained for most species (Table 3, Fig. 2). *B. longum* remain of high prevalence: 74% of the infants were colonized, 90% in the allergic group and only half of them in the healthy group. *B. bifidum*, *B. pseudocatenulatum* and *B. adolescentis* colonized respectively 42%, 42% and 26% of the

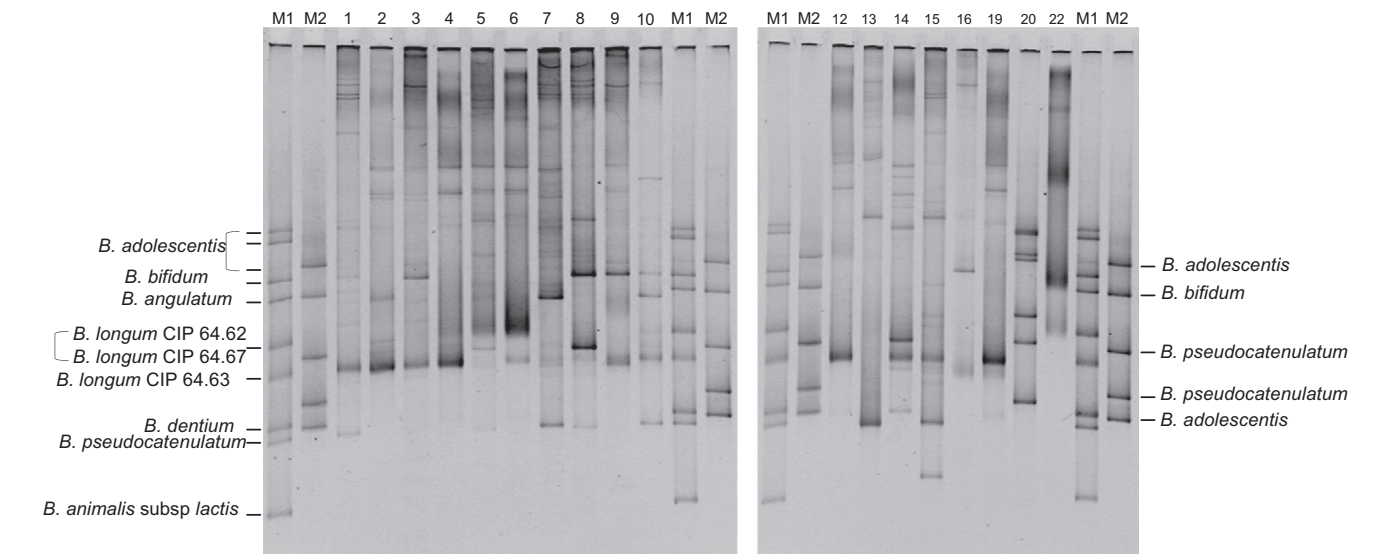


Fig. 2. TTGE profiles representing the bifidobacterial diversity of faecal samples from allergic (samples 1 to 10) and healthy (samples 12 to 22) infants. M1 and M2: standards consisting of a mixture of PCR products obtained from identified species or clones.

infants independently of allergy status and *B. dentium* was rare. However, *B. breve* colonized only 53% of the infants, whatever the group.

No statistical differences were observed between allergic and control infants. However, allergic infants tended to be more often colonized by the *B. longum* group compared with the control group ($p = 0.092$) as described by others [12,14]. We do not confirm the association previously reported between *B. adolescentis* and the *B. catenulatum/pseudocatenulatum* group and the allergy status [12,13,15]. This could be due to the fact that, although distribution of bifidobacterial species was in accordance with most studies, *B. adolescentis* was less isolated in our study than usually reported in infants [13,14], and in 5 year old children [16]. Indeed, the prevalence of *Bifidobacterium* species may vary with countries [37].

The literature shows numerous discrepancies concerning the link between bifidobacterial species and allergy. Rather than focus on the role for a single species, some authors study highlighted modifications of bifidobacterial diversity in allergic infants and children compared to healthy ones [16]. However controversial results were described [9,14–16,38]. In the present study, infants were colonized with 1–5 species (median 2 whatever the technique) independently of allergy status or age at sampling. Forty percent and 25% of the infants were colonized by at least 3 species using culture and TTGE, respectively. Although this is far less than in Sjogren et al. study (2009) in which 76% of Swedish infants aged 2 months were colonized by at least 3 species of *Bifidobacterium*, this confirms their results and the ones of Gore et al who did not observe any reduction in bifidobacterial diversity in term of species between allergic and non allergic infants [14,15].

No study evaluated the diversity of bifidobacterial colonization in infants in terms of strains. The use of a culture method associated with a fingerprinting molecular method allowed us to study *Bifidobacterium* isolates at a strain level. The 254 colonies studied (115 isolates from allergic infants and 135 from controls) corresponded to 60 different Box-PCR profiles, i.e. strains, with 15–20 bands per profile (Fig. 3). Two infants in each group were colonized by only one strain, and 2 others by two different strains but 60% were colonized by at least 3 strains. Seven infants (35%) were colonized by 2 strains of *B. breve* and 1 by two strains of *B. pseudocatenulatum*. Two control infants were colonized by more than one *B. longum* subsp. *longum* strain and 1 allergic one was colonized by two *B. longum* strains, respectively subsp. *longum* and subsp. *infantis*. Thus, strain diversity is high, amounting to 1 to 7 strains (median 3), independently of allergy status or age at sampling.

To conclude, our study shows that infants are colonized by several species and strains, including several strains from the same species. No differences in bifidobacterial diversity (either species or strain) were observed between allergic and control infants. For example, regression for species showed odd ratio of 1.99 (CI95%: 0.74–5.38) between allergic and non allergic which gives no evidence for a dependence between allergic status and the number of species. Trying to group the diversity in two classes (more or less than three or two species present) doesn't yield any significant result. The properties of intestinal bifidobacteria are highly strain-dependent [39], in particular concerning immunostimulatory properties [17,18]. Moreover, synergy and/or antagonism may occur within a complex microbiota, which might modify the impact of strains on immunity as observed in *in vitro* experiments [40]. Thus, the diversity in the bifidobacterial strains we observed in infants showed that the link between *Bifidobacterium* colonization and allergic diseases is complex and cannot be restricted to a single role for one species. One of the limitations of this study was the diversity of the clinical pattern of allergic infants. Restricting allergic infants to a homogeneous group of patients in terms of sensitization and of clinical appearance might yield more focused results.

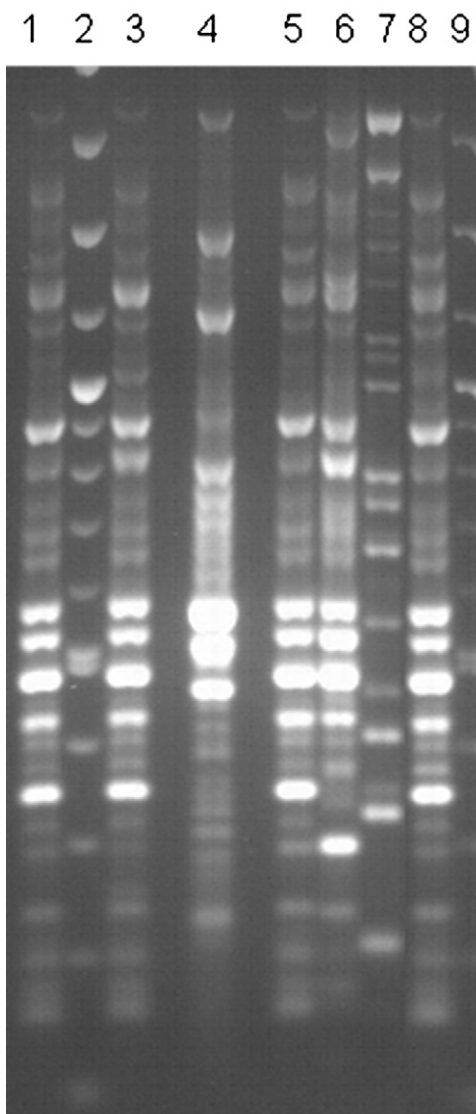


Fig. 3. Box-PCR fingerprinting. Example obtained for one infant. Each line corresponds to one isolated colony. Lines: 2 and 9: ladder; line 6: reference strain (*B. longum* NCC2705); infant's strains: lines 1, 3, 5, and 8: *B. longum*; line 4: *B. bifidum*, line 7: *B. breve*.

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