RESEARCH ARTICLE

Molecularly assessed shifts of *Bifidobacterium* ssp. and less diverse microbial communities are characteristic of 5-year-old allergic children

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Abstract

The composition of intestinal microbiota and the *Bifidobacterium* group community in 20 allergic and 20 nonallergic 5-year-old children was visualized by PCR-denaturing gradient gel electrophoresis (DGGE). The number of dominant bands in the DGGE profiles was smaller in allergic children than in nonallergic children ($P < 0.001$). Allergic children mainly formed a single group upon cluster analysis, whereas nonallergic children were divided between four different groups. In allergic children the *Bifidobacterium adolescentis* species prevailed, and in nonallergic children the *Bifidobacterium catenulatum/pseudocatenulatum* prevailed ($P = 0.01$ and $P = 0.01$, respectively). The less diverse composition of intestinal microbiota and prevalence of particular species of *Bifidobacterium* were characteristic of allergic children even at the age of 5 years.

Introduction

In the gastro-intestinal tract the microbiota consist of at least 10^{13} microorganisms and nearly 2000 mainly anaerobic species have been described (Moore & Holdeman, 1974; Zoetendal *et al.*, 1998; Macfarlane & Macfarlane, 2004; William, 2004). The development of microbiota starts from the very first days of life parallel with that of the immune system (Brandtzaeg *et al.*, 1989; Holt *et al.*, 2003). The gut has been considered the most important immune function-related organ as *c.* 70–80% of the immune cells of the body are present in the intestinal mucosa (Brandtzaeg *et al.*, 1989; Munoz-Lopez, 2004). The different components of intestinal microbiota stimulate the production of different cytokines and may enhance immune responses (Apperlo-Renkema *et al.*, 1993; Kimura *et al.*, 1997; Karlsson *et al.*, 2002). The increasing allergic disorders are multifactorial diseases, the aetiopathogenesis of which is correlated with the composition of gut microbiota (Björkstén *et al.*, 1999, 2001; Böttcher *et al.*, 2000; Watanabe *et al.*, 2003; Bischoff & Crowe, 2004; McKevith & Theobald, 2005)

The *Bifidobacterium* strains of the gastro-intestinal tract may differentially exert antimicrobial activity, suggesting their participation in the 'barrier effect' or 'colonization resistance' produced by indigenous microbiota (Liévin *et al.*, 2000). The faecal microbiota of breast-fed infants is usually predominated by *Bifidobacterium* forming up to 90% of the total faecal bacteria (Harmsen *et al.*, 2000). The low prevalence of bifidobacteria is characteristic for the infants who later develop allergy (Björkstén *et al.*, 1999, 2001; Kalliomäki *et al.*, 2001). The shifts in the prevalence of bifidobacteria in atopic and healthy adults have also been detected (Watanabe *et al.*, 2003). Moreover, the species composition of bifidobacteria in allergic infants differs from that of healthy children, the former being more like that of adults (Ouwehand *et al.*, 2001; Suzuki *et al.*, 2007). The shifts in prevalence and species composition of bifidobacteria point to the importance of the early immune stimulation by intestinal microbiota in the development of the allergic phenotype (Kimura *et al.*, 1997; Perdigon *et al.*, 2003; Young *et al.*, 2004). However, it is still under discussion whether these early

shifts in intestinal microbiota disappear or persist in preschool age (Sepp *et al.*, 2005).

The application of molecular analysis provides the possibility to assess the diversity of species composition of the microbiota putatively involved in the expression of allergy. Investigating rRNA/DNA or its corresponding genes by denaturing gradient gel electrophoresis (DGGE), followed by their cloning, sequencing and comparison with the database, is suggested for its rapidity and reliability (Zoetendal *et al.*, 2001; Tannock, 2001; Wang *et al.*, 2003). Information on the diversity of phylogenetically coherent bacterial groups, such as lactic acid bacteria or bifidobacteria, can be obtained using group-specific rather than domain-specific primers in PCR amplification (Heilig *et al.*, 2002; Murray *et al.*, 2005; Vaughan *et al.*, 2005).

Hence, the aim of this study was to compare the distribution of microbial populations and to investigate the predominant bifidobacterial species of the intestinal microbiota in allergic and nonallergic 5-year-old children using a molecular approach.

Materials and methods

Study group

The study group comprised 40 Estonian children, (17 M/23 F), who were at the age of 5 years randomly selected from a larger group ($n = 213$) in which the immune responses to allergens and the development of allergy were studied starting from birth (Julge *et al.*, 2001). The inclusion criteria were vaginal delivery, breast feeding at least till the end of the neonatal period, presence or absence of the diagnosis of allergy at the age of 5 years and availability of faecal samples. Twenty allergic [age: median 62.5 months (quartiles 60.5–64.0)] and 20 nonallergic [age: median 61.5 months (quartiles 61.0–63.0)] children were recruited. Nonallergic children had no signs of clinical allergy at any time during the first 5 years of life. The diagnosis of allergy was based on the clinical examination of the children and on the data obtained from the questionnaires (Julge *et al.*, 2001). Twelve of 20 allergic children had atopic dermatitis, seven had bronchial asthma and five had allergic rhinitis. Four of the children suffered simultaneously from two allergic manifestations (Table 1). Skin prick tests were performed, and blood sera and faecal samples were collected simultaneously from 20 allergic and 20 nonallergic 5-year-old children. For skin prick tests, fresh egg white and cow's milk, and extracts of house dust mite, cat, dog, birch and timothy were used (ALK Abello, Denmark). Skin prick tests were regarded as positive if the weal size, measured after 15 min, was 3 mm or more. The circulating IgE antibodies of the blood sera were determined against two food allergens (egg white and cow's milk) and six inhalants (house dust mite, cat, dog, birch, timothy and mugwort), employing a chemiluminescence method (Magic Lite SQ, ALK-Abello). A normal sensitivity method was used and the level of IgE antibodies was expressed in five classes (Julge *et al.*, 2001).

Ethical aspects

The Human Research Ethics Committee of the University of Tartu approved the study. Informed consent was obtained from the parents of the children.

Faecal samples and DNA extraction

Approximately 2 g of a fresh stool sample was placed in a plastic cup. The samples collected at home were kept in a domestic refrigerator at 4 °C for no more than 2 h before transportation to the laboratory, where the plastic cups were stored frozen at − 70 °C until use.

The DNA was extracted using QIAamp DNA Stool Mini Kit (Qiagen, Germany). The amount of DNA was determined visually after electrophoresis on a 1.2% agarose gel containing ethidium bromide (Zoetendal *et al.*, 2001).

Primers

All primers used in the study are targeted on the 16S RNA gene. Bacterial PCR products were produced with primers 968-GC-f and 1401-r (Lane, 1991), *Bifidobacterium* genus-specific PCR were performed with the Bif164-f and Bif662-r primers (Langendijk *et al.*, 1995; Satokari *et al.*, 2001). Im3-r and Im26-f were used for the construction of a clone library of 16S rRNA gene from *Bifidobacterium* ssp. (Kaufmann *et al.*, 1997). The primers T7 and SP6 labeled with IRD800 (Promega) were used for sequencing. All primers were purchased from MWG-Biotech (Ebensburg, Germany).

PCR amplification

PCR was performed in a reaction volume of 50 μL containing 10 mM deoxyribonucleotide triphosphate each, 1.25 U of *Taq* polymerase (Invitrogen, USA), 10X reaction buffer, 10 μmol of the each primer and 200 ng (1 μL) of DNA solution. Initial DNA denaturation and enzyme activation steps with the primers 968-GC-f and 1401-r were performed at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 20 s, and elongation at 68 °C for 40 s, which was followed by final elongation at 68 °C for 7 min. The annealing temperatures were set at 62 °C with primers Bif164-f and Bif662-GC-r, and at 57 °C with primers Im26-f and Im3-r.

DGGE analysis of PCR products

DGGE analysis of PCR amplicons was performed using a Dcode™ System apparatus (Bio-Rad, Hercules, CA).

Table 1. Cluster analysis of allergic (A) and nonallergic (H) children at 5 years of age on the basis of health and immunological aspects

	Clinical data			Clustering analysis	
No. of children A/H	Diagnosis	SPT	IgE antibody class	Cluster	Group
A13	BA, AR	Neg.	CM-2, EW-1		NG
A4	BA	Neg.	CM-1, EW-1, HDM-2, birch-1	A1	**A**
A17	AD, AR	Cat, dog, birch	Cat-5, dog-4, birch-4		
A5	AD	Neg.	EW-1	A2	
A6	AD, BA	ND	ND		
A3	AD	Timothy	Timothy-1		
A7, A11	AD, AD	Neg.	Neg.	A3	
A1	AR	Neg.	Dog-3		
A18	AR	Neg.	CM-1, HDM-1, cat-1		
A15	BA	Neg.	Timothy-1	A4	
A19	AD	Neg.	CM-1, dog-1, timothy-1		
A8	AD, AR	Dog	CM-1, dog-1, timothy-1	A5	
A10	AD	Neg.	CM-1, EW-1, HDM-1, birch-2, timothy-1		
A20	AD	Neg.	CM-2		
A2	BA	Dog, cat	Cat-2	NC	
A9	AD	Neg.	Neg.		
A14	AD	Cat, birch	Birch-2		
H1, H2, H3	No	Neg.	Neg.	H1	
H11, H12	No	Neg.	Neg.	H2	
H5	No	Neg.	Dog-1	NC	
H10, H13, H14	No	Neg.	Neg.		
A12	BA	HDM	EW-1, HDM-4, birch-2	NC	**B**
H7, H8	No	Neg.	Neg.		
H9	No	Neg.	CM-1, EW-1, dog-1, HDM-1, birch-1, timothy-1	H3	**C**
H6	No	Neg.	Neg.		
H15, H16, H18, H19	No	Neg.	Neg.	H4	**D**
H4	No	Neg.	CM-1, EW-1, cat-2, dog-2, HDM-1, birch-1, timothy-1		
H17, H20	No	Neg.	Neg.	NC	
A16	BA	Neg.	CM-1		

AD, atopic dermatitis; AR, allergic rhinitis; BA, bronchial asthma; SPT, skin prick test; CM, cow's milk; EW, egg white; HDM; house dust mite; NG, no group; ND, not detected; NC, not clustered, Neg., negative.

Polyacrylamide gels (8% w/v) acrylamide–bisacrylamide (37.5 : 1) in 0.5 × Tris-acetic acid-EDTA buffers with a denaturing gradient were prepared with a gradient mixer and Econopump (Bio-Rad). Gradients from 30% to 60% were employed for the separation of the products amplified with universal primers and from 45% to 60% for the products amplified with primers specific for *Bifidobacterium* ssp.

Cloning of PCR products

The PCR amplicons were purified and concentrated with the QIAquick PCR purification kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and cloned in *Escherichia coli* JM109 using the pGEM-T vector system (Promega, Madison, WI). Colonies for sequencing were selected according to the migration position of the PCR fragment of the clone in DGGE in comparison with the fragments in the original DGGE profile. The plasmid DNA of the selected transformants was isolated using the QIAprep spin miniprep kit (Qiagen).

Sequence analysis

Sequencing of the cloned PCR fragments was carried out using purified plasmid DNA and the sequencing primers SP6 and T7. Sequencing reactions were performed with Sequenase sequencing kit (Amersham, Slough, UK) according to manufacturer's instructions. The sequences were analyzed with the automatic LI-COR DNA Sequencer 4000 L (Lincoln, US) and corrected manually. Sequence alignment of the complementary strands was carried out using the DNASTAR SEQMAN program (Madison, WI). Similarity searches for the 16S rRNA gene sequences were performed in the GenBank database using the BLAST algorithm.

Statistical analysis and calculation of similarity indices

The statistical analyses were performed using the SIGMASTAT (Jandel Scientific, USA) and EXCEL (Microsoft Corp.) software programs. DGGE gels were scanned and the software of BIONUMETRICS 2.5 (Applied Maths, Belgium) was employed

for comparing the DGGE profiles, based on Dice's similarity coefficient, and for analyzing the gel diversity in allergic and nonallergic children (Fromin *et al.*, 2002). The Odds ratio (OR) test with 95% confidence intervals (CI 95%), descriptive statistics (quartiles 25–75%), Fisher and χ^2 tests were used for the statistical analysis of comparison between the number of bands of total microbiota and the species of bifidobacteria. For comparison of the similarity indices of the DGGE profiles, Student's *t*-test was used or the Mann–Whitney rank sum test, 'STATGRAPHICS' (Statistical Graphics Corp., USA).

Results

Atopic sensitization of children

In allergic children the breast milk was supplemented earlier with cow milk than in the nonallergic children (range: 1–46 weeks, median 5 weeks vs. range: 1–40 weeks; median 13 weeks; $P = 0.004$).

The clinical data of children are presented in Table 1. The skin prick test was positive in six allergic children and negative in all nonallergic children. The IgE antibodies against various allergens were detected in 16 clinically allergic children. Three healthy children had low levels of allergen-specific circulating IgE. In particular, the IgE antibodies against cow milk (CM) were found in eight allergic and two nonallergic children. No correlation was found between presence of cow milk IgE and the time of supplementation with cow milk.

Comparison of dominant bacterial DGGE profiles of allergic and nonallergic children

Complex profiles were obtained consisting of multiple amplicons, each assumed to represent a unique bacterial 16S rRNA gene sequence (Fig. 1). The number of abundant bands of the DGGE profiles obtained from the 20 allergic children varied from 15 to 24, whereas the number of such bands in the 20 nonallergic children varied from 17 to 36 [median 17.5 (quartiles 16.0–20.5) vs. 23.5 (quartiles 20.5–27.0); $P < 0.001$, respectively].

The similarity indices (SI) of the banding patterns of the DGGE gels obtained from allergic and nonallergic children

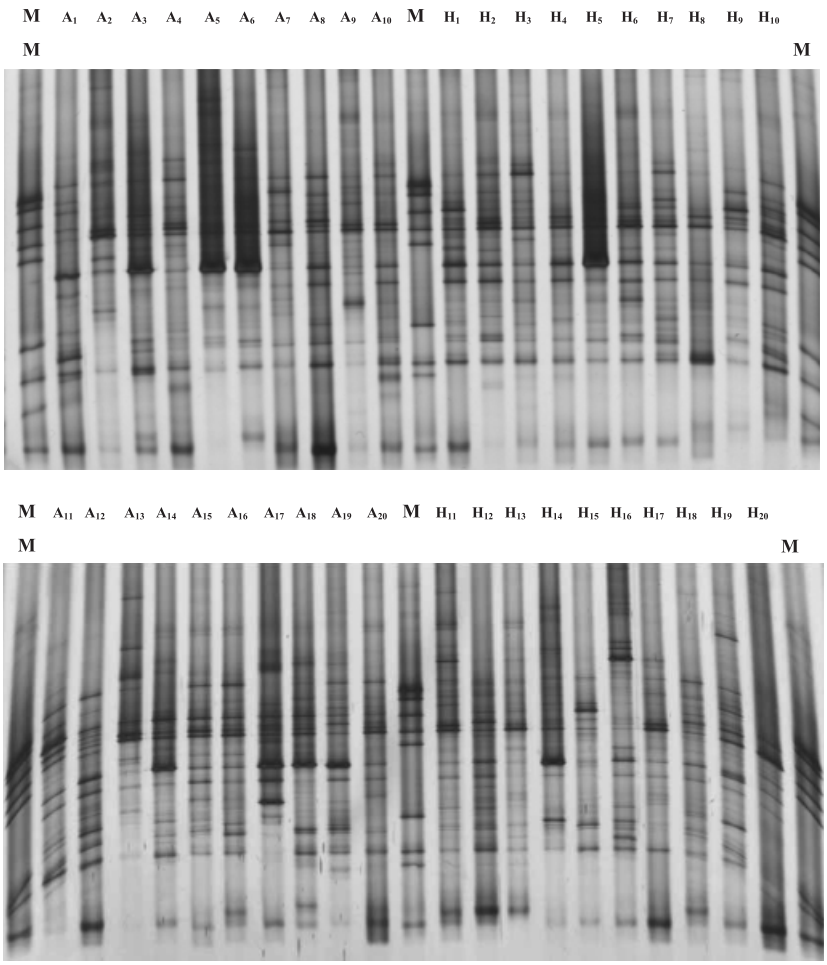

Fig. 1. DGGE analysis of the amplified V6–V8 regions of the 16S rRNA gene of faecal samples of allergic (A_1–A_{20}) and nonallergic (H_1–H_{20}) children. M indicates the marker for DGGE.

were calculated. No statistically significant difference was found between the dominant profiles of these two groups of children: in the mean index was 65.4 ± 16.0 in allergic children and 63.7 ± 13.2 in nonallergic children ($P = 0.755$).

To find out whether the similarity or difference in the dominant DGGE gel tracks could be attributed to the allergy, a cluster analysis was performed using a distance matrix (UMPAG) algorithm. In total, 39 of 40 samples (97.5%) of both allergic and nonallergic children were classified into four groups (A–D) on the basis of their DGGE patterns with a similarity of $\geq 80\%$ (Fig. 2).

For allergic children, 17 of 20 samples belonged to group A, forming five clusters (A1–A5), two samples belonged to groups B and D, and one sample (A13) did not belong to any group. The 20 samples from nonallergic children belonged to four different groups (A–D); in group A they formed two clusters (H1, H2) and in groups C and D one cluster each (H3 and H4, respectively).

No significant association was found between the results of the clustering of 16S rRNA gene amplicons and the grouping based on the clinical diagnosis. However, six of 12 patients with atopic dermatitis belonged to the particular clusters (A2 and A5; see Table 1 and Fig. 2).

Analysis of the diversity of the bifidobacterial community by PCR-DGGE

No differences were found in the prevalence of *Bifidobacterium* ssp. between allergic and healthy children (20/20). To compare the complex bifidobacterial communities occurring in the faecal samples of the cohort, *Bifidobacterium* genus-specific PCR in combination with DGGE, cloning and sequencing of the specific PCR fragments was applied. Bifidobacteria were detected in all samples of allergic and nonallergic children. Bifidobacterial profiles varied from three to six dominant bands mostly individually different in detected lines of the DGGE profile.

The sequence analysis of the 16S rRNA gene amplicons showed a clear difference in the composition of the faecal bifidobacterial species in allergic and nonallergic children. A total of 128 bifidobacterial clones (55 from allergic and 73 from nonallergic children) were subjected to sequence analysis. The number of bifidobacteria species was not associated either with breast feeding or supplementation with cow milk. Comparison of the obtained sequences with those of the database revealed a significant degree of similarity of the fragments (97–100%) to the 16S rRNA gene sequences of known *Bifidobacterium* species. *Bifidobacterium adolescentis* was found in the faecal samples of 14 (70 %) allergic children and in only five (25 %) nonallergic children [Table 2; 14/6 vs. 5/15; OR = 5.8 (95% CI 1.7–19.7); $P = 0.01$] whereas a significantly higher number of individual clones of *B. adolescentis* were present in allergic children (18/55 vs. 5/73; $P < 0.001$). At the same time, in nonallergic children the *Bifidobacterium catenulatum/pseudocatenulatum* group prevailed (14/6 vs. 5/15; $P = 0.01$), showing a higher number of different clones as compared with allergic children (23/73 vs. 5/55; $P = 0.005$).

Discussion

Using DGGE, cloning and sequencing of 16S rRNA gene amplicons, we found in this study that the distinct pattern of intestinal microbiota previously assessed by culture-based methods persists in clinically allergic children at the age of 5 years.

In allergic children the number of dominant bands in the individual DGGE profiles was significantly lower than in nonallergic children, indicating a less diverse composition of their intestinal microbiota. By culture-based methods, 5-year-old Estonian children born in the early 1990s had significantly higher counts of anaerobes such as *Bifidobacterium*, *Bacteroides*, *Eubacterium* and *Peptostreptococcus* than their counterparts born later (Sepp *et al.*, 2006). At the same time, the prevalence of allergic diseases was low, whereas now it has increased in Estonia (Björksten *et al.*, 1998; Raukas-Kivioja *et al.*, 2003; Voor, 2006). These findings make it necessary to modify the hygiene hypothesis that relates the reduced exposure to infections and more hygienic lifestyle to the rapid increase of allergic diseases in industrialized countries (Strachan, 1989). It is more probable that it is not the reduced contact with infectious agents, but the less diverse normal microbiota expressing low microbial pressure in childhood that is responsible for the development of allergy. This process seems to be associated with the absence of tolerance induction or down-regulation of sensibilization to various nonharmful foods or inhaled allergens, as postulated by Brandtzaeg (2002).

The distinct pattern of the intestinal microbiota of allergic children was also seen from calculation of the distance matrix of their DGGE pattern. The microbiota of both nonallergic and allergic children were allocated into four groups (A–D) with a similarity of $\geq 80\%$. The most striking difference was that 85% (17 of 20) of the samples of allergic children with different clinical manifestations fell into group A with five different clusters. The samples from nonallergic children, by contrast, were allocated evenly between A–D groups. Similarly, in some other diseases like ulcerative colitis and Crohn's disease, the particular and less diverse microbiota have been found using molecular methods (Zoetendal *et al.*, 2002; Seksik *et al.*, 2003; Ott *et al.*, 2004; Ben-Amor, 2005).

We could not see any differences in the prevalence of *Bifidobacterium* sp. between allergic and healthy children, earlier assessed by culture-based methods (Björkstén *et al.*, 1999; 2001; Sepp *et al.*, 2005). However, using specific

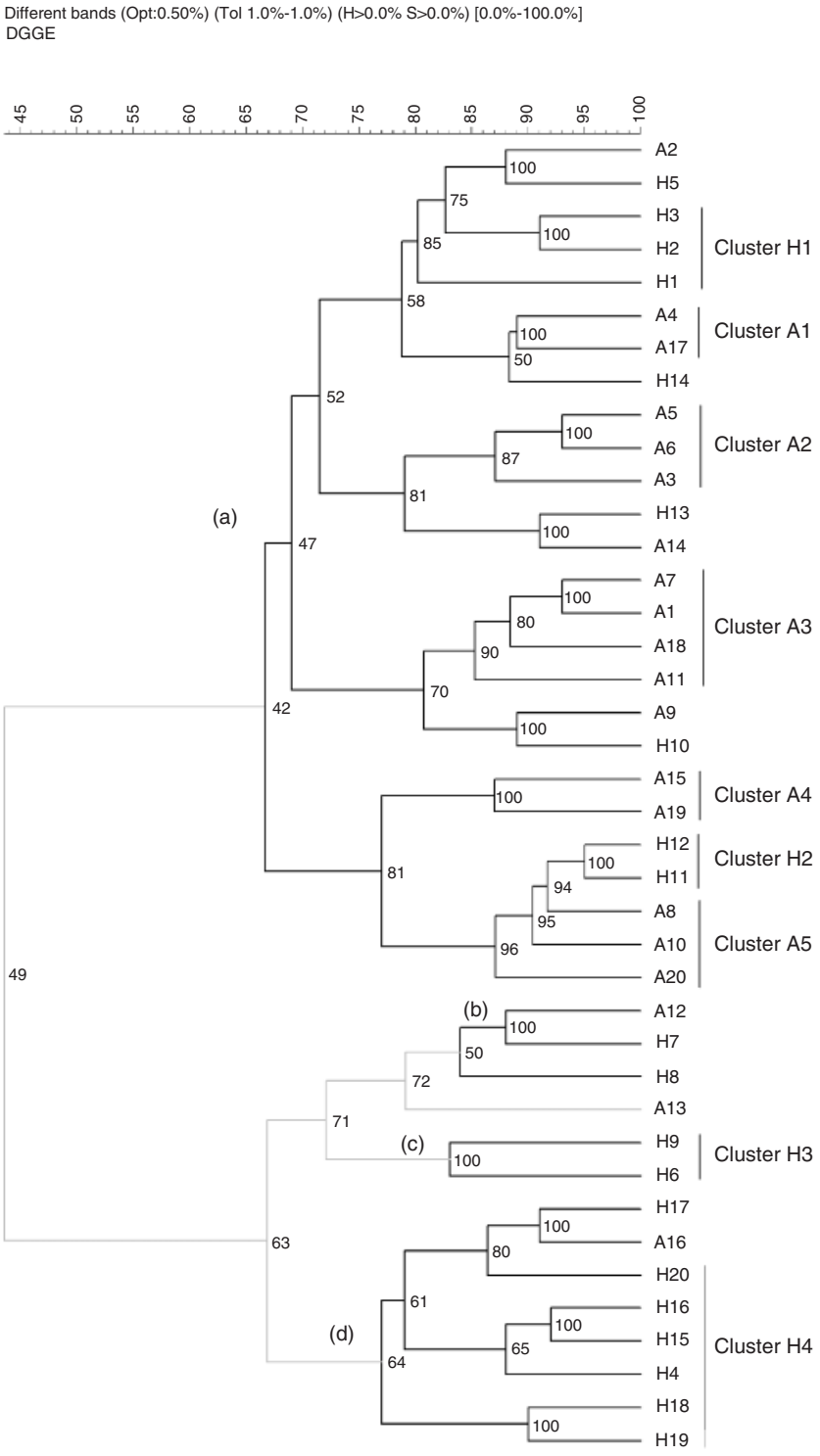

Fig. 2. UPGMA dendogram illustrating the correlation between different DGGE profiles of allergic (A) and nonallergic (H) children.

Bifidobacterium primers we observed significant differences in the distribution of *Bifidobacterium* species: allergic children were mainly colonized with *B. adolescentis*, whereas in nonallergic children the *B. catenulatum/pseudocatenulatum* group was predominant. These data correspond well to the results reported by Ouwehand and coworkers, who showed using cultural methods that 50% of the total bifidobacterial isolates of allergic children were *B. adolescentis* (He *et al.*,

Table 2. Number of different species and clones of *Bifidobacterium* in allergic and nonallergic children

Child	Allergic								Nonallergic							
Species	*B. adolescentis*	*B. catenulatum/ pseudocatenulatum*	*B. longum*	*B. bifidum*	*B. breve*	*B. angulatum*	*B. dentium*	Total	*B. adolescentis*	*B. catenulatum/ pseudocatenulatum*	*B. longum*	*B. bifidum*	*B. breve*	*B. angulatum*	*B. dentium*	Total
1	•		•	•						•	•					
2	•					•				•	•					
3	••									•	•					
4	•	•	•						•	•	•			•		
5			••							••	••			•	•	
6			••						•		•			•		
7	•	•	•	••						••	••					
8			•							•	••					
9	•	•							•		•					
10	•					•				••	•	•				
11			•							•	•					
12			•••								••					
13	••	•	••								•					
14	••		•••							••	•		•	•		
15	•		•							•••	••		•			
16	•		•							•••	•		•	•		
17	••	•	••		•					••	••		•	•		
18	•		•		•				•	•	••			•		
19			•								••		•	•		
20	•		•			•			•	••	••			•		
Sum of positive subjects/total clones	14[†]/18*	5/5	16/24	2/3	2/2	3/3	0/0	**20/55**	5/5	14[‡]/23[#]	20/29	1/1	5/5	9/9	1/1	**20/73**

•Bifidobacterial species.

[†]$P = 0.01$;

[‡]$P = 0.01$.

Sequenced bifidobacterial clones $n = 128$;

*$P < 0.001$;

[#]$P = 0.005$.

2001; Ouwehand *et al*., 2001). The culture-independent methods have shown that the *B. catenulatum/pseudocatenulam* group was the most common species in the adult intestinal tract, followed by *Bifidobacterium longum, B. adolescentis* and *Bifidobacterium bifidum*, whereas *Bifidobacterium breve*, *Bifidobacterium infantis* and *B. longum* are the predominant species in infants (Matsuki *et al*., 1999, 2004). *Bifidobacterium adolescentis* that prevails in allergy is not easily detectable by culture, yet it can be revealed by PCR and sequencing (Matsuki *et al*., 1999). A recent study using flow cytometry has confirmed that *B. adolescentis*-related species are likely to be dead or damaged by the time faecal samples are passed to the laboratory (Ben-Amor *et al*., 2005).

The number of species of bifidobacteria was not related to the feeding method of used for the infants, although in allergic children cow's milk supplementation was begun earlier. The same tendency has been revealed at the age 1–6 months where the differences in the bifidobacteria species were not related to the feeding method (Suzuki *et al*., 2007).

It is difficult to explain why at the age of 5 years the species composition of bifidobacteria in allergic children differs from that in healthy children of the same age and also from the microbial communities of adult persons. Different lactic acid bacteria are able to modulate the immune response of the host by increasing IgA synthesis and by inducing different pro- and anti-inflammatory cytokine production (Yasui *et al*., 1995; Kimura *et al*., 1997; Pessi *et al*., 2000; Perdigon *et al*., 2003). The species *B. adolescentis* prevailing in allergic children more effectively triggers the proinflammatory cytokines tumour necrosis factor (TNF)-α, interleukin (IL)-6 and IL-12, but it is not able to induce the regulatory cytokine IL-10, which is an important anti-allergic immune response (Kramer *et al*., 1995; He *et al*., 2002). In contrast, some other species such as *B. bifidum, B. longum* and *B. catenulatum/pseudocatenulatum* induce the production of the regulatory cytokine IL-10 (Young *et al*., 2004). Moreover, *B. adolescentis* strains isolated from allergic infants adhered not well to the human intestinal mucus, which may be related to the aberrant immune responses (He *et al*., 2002).

Several studies have indicated that supplementation of children's diet with probiotic lactobacilli and bifidobacteria, such as *Lactobacillus* GG and *Bifidobacterium lactis* Bb-12, can somewhat reduce allergic disorders in atopic children and may have a strong impact on development, microbial cross-talk, evolution and modulation of a microbiota (Mattila-Sandholm *et al*., 1999; Isolauri *et al*., 2000; Kirjavainen *et al*., 2002; Guemonde *et al*., 2006). For alleviation of allergic disease, it is tempting to suggest the consumption of some probiotic of *B. catenulatum/pseudocatenulateum*, the species present in healthy, but absent in allergic, children.

In conclusion, the molecular study confirms the persistence of the less diverse composition of the intestinal microbiota and the prevalence of particular species of *Bifidobacterium* in clinically allergic children even at the age of 5 years.

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