

Fecal microbiota in sensitized wheezy and non-sensitized non-wheezy children: a nested case–control study

C. S. Murray*, G. W. Tannock†, M. A. Simon†, H. J. M. Harmsen‡, G. W. Welling‡, A. Custovic* and A. Woodcock*

*North West Lung Centre, Wythenshawe Hospital, Manchester, UK, †Department of Microbiology, University of Otago, Otago, New Zealand and ‡Department of Medical Microbiology, University of Groningen, Groningen, The Netherlands

Summary

Background It has been suggested that intestinal microbiota of allergic and non-allergic children differs in composition, and that microbiota–immune system interactions may predispose children to develop sensitization. Previous studies have examined fecal microbiota of allergic children with atopic dermatitis, but little is known about that of atopic wheezy children.

Objective To investigate the composition of the fecal microbiota of young sensitized wheezy and non-sensitized non-wheezy children, using molecular methods.

Methods Within the context of a prospective birth cohort, we carried out a nested case–control study of sensitized wheezy children (cases) and non-sensitized non-wheezy controls. Cases and controls were matched for age, sex, parental atopy, allergen exposure, and pet ownership. We evaluated the composition of fecal microbiota by nucleic acid-based methods (PCR combined with denaturing gradient gel electrophoresis and quantification of bifidobacteria by fluorescent *in situ* hybridization).

Results Thirty-three case–control pairs (mean age 4.4 years) provided stool samples. Comparison of total bacterial community profiles showed that each child had a unique fecal microbiota (mean Dice's similarity coefficient 22%, range 3.3–60.8%). There was no difference between the groups in prevalence of Lactic Acid bacteria (12/33 vs. 11/33, $P = 0.8$) or bifidobacteria (30/33 vs. 31/33, $P = 1.00$, cases vs. controls). The bifidobacterial species detected were similar in both groups. The percentage of bifidobacteria in total fecal microflora was no different between cases (median 1.7%, range 0–20.8%) and controls (1.9%, 0–18.2%, $P = 0.7$). However, cases with eczema had significantly fewer bifidobacteria (median 1.6%, range 0–4.8%) than their controls (4.0%, 1.9–18.2%, $P = 0.05$).

Conclusion We found no differences in fecal microbiota composition between sensitized wheezy and non-sensitized, non-wheezy children aged 3–5 years using nucleic acid-based methods. Differences appear to be isolated to those allergic children with eczema.

Keywords atopic sensitization, atopy, children, eczema, fecal microbiota, wheeze

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Introduction

The gut microbiota provides a continuous stimulation of the immune system commencing in infancy and may play a crucial role in the maturation of the immune system [1]. Some *Lactobacillus* strains have been shown to induce IL-12 and INF- γ production and thus suppress total and specific IgE secretion [2–4]. In addition, *Lactobacillus* species may inhibit IL-4 and IL-5 secretion [5]. It has recently been demonstrated that some, but not all species of bifidobacteria stimulate IL-10 production by dendritic cells harvested from cord blood [6].

Over recent years differences have been documented in the composition of the fecal microbiota between healthy infants

in countries with a low and a high prevalence of allergy [1, 7], and between allergic and non-allergic individuals [8, 9]. Furthermore, it has been demonstrated that these differences precede the development of sensitization and clinical manifestations of allergic disease [10, 11]. Differences in gut microbiota between allergic and non-allergic individuals have also been suggested in studies using indirect indices of gut microbiota (e.g. fatty acid content of stool, etc.) [12–14].

Studies investigating fecal microbiota differences have previously focused on children with eczema rather than those with wheeze. In addition, studies using probiotic preparations in order to alter gut microflora and thus potentially modify allergic disease have all been carried out in children with eczema with and without food allergy [15, 16].

We used PCR combined with denaturing gradient gel electrophoresis (PCR-DGGE) and fluorescent *in situ* hybridization (FISH) to investigate the composition of the fecal

Correspondence: Clare S. Murray, North West Lung Centre, Wythenshawe Hospital, Manchester M23 9LT, UK.
E-mail: cmurray@fs1.with.man.ac.uk

microbiota of young sensitized wheezy and non-sensitized non-wheezy children.

Methods

Study population

Within the context of a prospective birth cohort study (National Asthma Campaign Manchester Asthma and Allergy Study, NACMAAS), we carried out a nested case-control study comparing fecal microbiota composition of wheezy sensitized and non-wheezy non-sensitized children. The cohort is described in detail elsewhere [17–19]. Briefly, the subjects were recruited *in utero* (8–10th week of pregnancy) by screening parents in the ante-natal clinic using skin testing and a questionnaire regarding allergic diseases. During the first 3 years of life parents were asked to keep diary cards regarding their child's health. All participants were invited to attend a review clinic at the age of 3 years (± 4 weeks). A standard respiratory questionnaire was interviewer administered [20]. Children were skin prick tested (*Dermatophagoides pteronyssinus*, cat, dog, mixed grasses, egg, milk, positive and negative control; Bayer, Elkhart, IN, USA). Sensitization was defined as a weal at least 3 mm greater than the negative control. Following the 3-year follow-up visit parents were invited to enter their children into the case-control study. Homes were visited at birth and age 3 years and dust samples from the child's mattress and the lounge floor were collected and extracted. Mite, cat and dog allergens were assayed using monoclonal antibody-based enzyme-linked immunoassay [21].

Based on the data from the 3-year follow-up, cases were defined using the following criteria:

- sensitized (skin test positive to at least one allergen);
- recurrent wheeze (at least three parentally reported wheezy episodes).

cases were matched with control children (non-sensitized, i.e. skin test negative, no history of wheeze) according to the following variables:

- parental atopic status (skin prick test results and symptoms of disease);
- sex;
- month of birth;
- pet ownership;
- use of environmental control measures in early life.

Assessment of gut microbiota

The subjects provided a stool specimen, which was immediately refrigerated and then collected from the subject's house within 2 h. The sample was transported to the laboratory on dry ice and stored at -80°C until analysis.

Extraction of bacterial DNA from feces, polymerase chain reaction, and denaturing gradient gel electrophoresis

The methods for the extraction of bacterial DNA from feces, the amplification of target bacterial sequences, and the generation of microbiota profiles have been described

Table 1. Primers and probes used in the analysis of the fecal microbiota

Primer/probe	Nucleotide base sequence
HDA1-GC	5'-GC clamp-ACT CCT ACG GGA GGC AGC AGT-3'
HDA2	5'-GTA TTA CCG CGG CTG CTG GCA C-3'
LAC1	5'-AGC AGT AGG GAA TCT TCC A-3'
LAC2-GC	5'-GC clamp-ATT YCA CCG CTA CAC ATG-3'
ForTal	5'-CGT CGC CTT CTT CTT CGT CTC-3'
RevTal-GC	5'-GC clamp-CTT CTC CGG CAT GGT GTT GAC-3'
Bif164	5'-CAT CCG GCA TTA ACC ACC C-3'
GC clamp	5'-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG G-3'

previously [22]. Fecal microbiota profiles were generated using universal bacterial PCR primers (HDA primers) as well as profiles of the lactic acid bacteria (including lactobacilli) and *Bifidobacterium* populations in the feces (Lac primers, Tal primers) [23–25]. Identification of bacterial species represented in the DGGE profiles was carried with reference to identification ladders or by elution of DNA fragments from the gels, cloning and sequencing [22–24]. The primers and probe sequences are given in Table 1.

Quantification of bifidobacteria in fecal samples

Enumeration (expressed as percentage of total fecal microbiota) of bifidobacteria was achieved by FISH using the *Bifidobacterium* genus-specific probe, Bif164. Total numbers of microbial cells (total microbiota) were obtained by 4',6-diamino-2-phenylindole staining [26].

Statistical analysis

Total bacterial community (HDA) profiles were compared using Quantity One version 4.2 software, which is part of the Discovery Series (Bio-Rad Laboratories, Hercules, CA, USA), and were performed using Dice's similarity coefficient analysis. The rest of the statistical analysis was carried out using SPSS for Windows, version 9.0. Since counts were not normally distributed, non-parametric tests (Wilcoxon's signed ranks test) were used to compare atopic and non-atopic children. Demographic data were normally distributed and paired samples *t*-test was used for analysis.

Results

Of 541 children reviewed at age 3 years, 43 were skin test positive at age 3 years and had a history of parentally reported wheeze in the first 3 years of life. Of these, 37 parents agreed to take part in this study. All 37 (20 males) were matched with a non-sensitized, non-wheezy control subject. Stool samples were obtained on 33 matched pairs.

Demographics

There were no significant differences between the groups in age (mean, 95% confidence intervals; 4.40 years, 4.27–4.54 vs. 4.39 years, 4.26–4.54, $P = 0.94$), height (mean, 95% confidence intervals; 104.2 cm, 102.5–105.8 vs. 104.5 cm, 102.9–

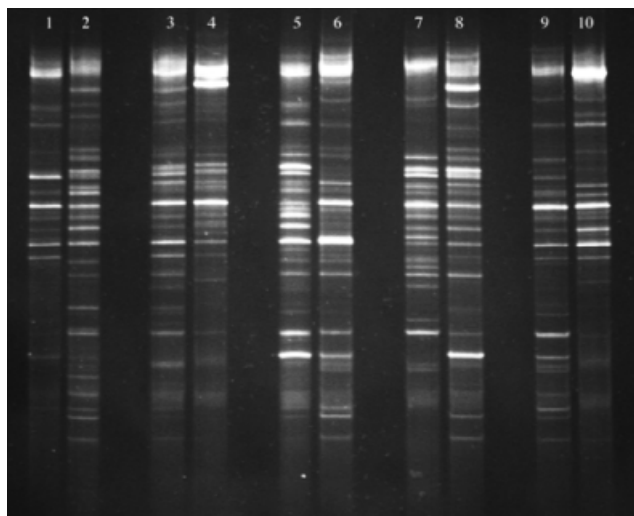


Fig. 1. Examples of denaturing gradient gel electrophoresis profiles generated from the fecal microflora of children. Lanes 1, 3, 5, 7, and 9 are samples from cases; lanes 2, 4, 6, 8, and 10 are samples from matched controls.

106.2, $P = 0.76$) or weight (17.7 kg, 16.7–18.7 vs. 18.0, 17.2–18.9, $P = 0.56$, cases and controls, respectively). Fourteen children in each group had a pet cat, dog or both. There were no significant differences in pet allergen levels in the children's homes. Dust samples collected at 3 years of age from the child's mattress had similar levels of Der p 1 in both groups (data not shown, available on request). There was no significant difference in the number of cases and controls born by vaginal delivery (22 vs. 27, respectively, $P = 0.26$). There was no significant difference in the number of cases and controls whose mothers were current smokers (8 vs. 4, respectively, $P = 0.34$).

Fecal microbiota

Fecal microbiota profiles Comparison of total bacterial community profiles generated from fecal DNA by PCR/DGGE using HDA primers showed that the microbiota of each child was unique. Dice's similarity coefficients averaged 22.0% and ranged from 3.3% to 60.8%. Examples of microbiota profiles from matched cases and controls are shown in Fig. 1.

Detection of lactic acid bacteria The frequency of detection of lactic acid bacteria (Lac PCR primers/DGGE) did not differ between cases (12/33) and controls (11/33, $P = 0.8$). Because of the low frequency of detection of these bacteria, identification of species was not made.

Detection of bifidobacterial species The prevalence of bifidobacterial species in the feces (Tal PCR primers/DGGE) did not differ between cases (30/33) and controls (31/33, $P = 1.00$, Fisher's exact test). The range of bifidobacterial species detected in cases and controls was similar in both groups (Table 2).

Quantification of bifidobacteria in feces The proportion that bifidobacteria comprised of the total fecal microbiota

Table 2. The composition of the *Bifidobacterium* population in cases and controls

<i>Bifidobacterium</i> species	Cases	Control	<i>P</i> -value
<i>B. adolescentis</i>	28 (85%)	30 (91%)	0.45*
<i>B. bifidum</i>	4 (12%)	5 (15%)	1.0†
<i>B. longum</i>	13 (39%)	12 (36%)	0.80*
<i>B. catenulatum</i>	1 (3%)	1 (3%)	1.0†
<i>B. pseudocatenulatum</i>	2 (6%)	2 (6%)	1.0†

* χ^2 test. †Fisher exact test.

was no different between cases (median 1.71%, range 0–20.83%) and controls (median 1.88%, range 0–18.23%, $P = 0.73$, Wilcoxon's signed ranks test).

Sub-analysis Eight of the wheezy atopic cases also had eczema. There was no difference in the prevalence of lactic acid bacteria or bifidobacterial species in the feces for wheezy atopic cases with eczema compared with their controls. However, the proportion of the total fecal microbiota that was comprised of bifidobacteria was significantly lower in the cases with eczema (median 1.6%, range 0–4.8%) compared with their controls (median 4.0%, range 0–18.2%, $P = 0.05$, Wilcoxon's signed ranks test).

Discussion

In this case-control study, the application of nucleic acid-based methods, established as an accurate method for the analysis of complex bacterial communities [27, 28], did not demonstrate any difference in the prevalence of either lactic acid bacteria (including lactobacilli) or bifidobacteria in the feces of sensitized pre-school children with a history of recurrent wheeze and non-sensitized, non-wheezy controls. In addition, the range of bifidobacterial species and the proportion that bifidobacteria comprised of the total fecal microbiota was very similar between the two groups. Moreover, the uniqueness of human fecal microbiota that has been demonstrated in adults [29] has been shown by our results to extend to that of children. This uniqueness confounds studies of the impact of the gut microbiota on disease processes [30].

Our findings differ from previously published data in studies where bacteriological culture of feces was used [1, 8, 11]. The types of bacteria that are detected by bacteriological culture are highly dependent on the culture medium used and provision of adequate anaerobic conditions [28]. Approximately 60% of the human fecal microflora has not yet been cultivated under laboratory conditions, even when optimal anaerobic conditions and freshly collected feces are used [25]. It is noteworthy that fecal samples in several previous studies were frozen prior to culture, which would favour the detection of bacteria that survived during storage and freeze-thaw, while biasing against the detection of species that would not survive freeze-thaw. Fortunately, adequate analysis of the fecal microflora can be carried out by the use of nucleic acid-based methods [31]. Bacterial species present in the sample, even if uncultivable under laboratory conditions, can be detected and quantified, and the proportions of

bacterial groups remain stable in samples stored at -20 to -80°C [27, 28]. Thus, the nucleic acid-based techniques used in this study provide more appropriate methodologies to compare fecal microbiota.

Apart from the methods used for comparing the bacterial communities, the other strengths of our study were the very careful definition of the outcomes and matching of cases and controls according to a number of factors, which are associated with the development of atopic sensitization and wheeze (parental allergic status assessed by skin testing and symptoms of disease, gender, month of birth, pet ownership and mite allergen exposure). If differences in gut microbiota composition truly exist in very early life between children who go on to develop atopic sensitization compared with those who do not, it is possible that these differences are genetically determined. Zoetendal et al. [32] reported that the fecal microbiota profiles of monozygotic twins were more similar than when comparisons were made between siblings. It may be that there are genetically influenced differences in gut permeability or other physiological factors that provide an altered gut ecosystem that might predispose children towards development of sensitization. Thus, early differences in intestinal microbiota could be a consequence of a genetic predisposition towards atopy, rather than the cause of disease, or alternatively a genetic predisposition towards the growth of certain types of microbiota may predispose to atopic disease. Careful matching of cases and controls according to parental sensitization and symptoms of allergic disease takes this possibility into account.

Unfortunately, although we were able to match our cases and controls for many factors, which are considered to be risk factors for allergic disease, we were unable to match for all the factors which may influence the establishment of the gut microbiota, such as mode of delivery and antibiotic usage. This is clearly a weakness of the study. However, it is worth noting that we found no difference in the proportion of children born by caesarian section between cases and controls.

We set out to investigate whether any differences existed between atopic wheezy children and non-atopic non-wheezy children. The results suggest that no such differences exist. Although the number of children with eczema in our study is small, it appears that there may be a difference in fecal microbiota confined to children with eczema. We found results similar to other studies in this small but well-defined population, i.e. significantly fewer bifidobacteria as a total of the bacterial community. Kalliomaki et al. [10] found using nucleic acid-based methods that atopic children had fewer clostridia and tended to have fewer bifidobacteria at 3 weeks of age, but not by 3 months compared with non-atopic children. In this study approximately 50% of atopic children had eczema, and it is possible that this has had a bearing on the results.

Given the diversity of the phenotype of allergic disease (i.e. any sensitized individual may have no clinical disease, or any combination of atopic dermatitis, allergic rhinitis and/or asthma), it is possible that gut microbiota plays differing roles in children with different allergic disorders.

Most published studies have examined fecal microbiota composition of children in the first year or two of life [1, 8, 10, 11]. The children in our study had a median age of 4.4 years

and it is possible that differences in microbiota occur only at an earlier age. However, a recent study from Japan, found that children with eczema, who were a mean age of 7.9 years had significantly lower counts of bifidobacteria than their age-matched controls [33]. The possibility that differences do occur in early life in children who go on to develop atopic wheeze clearly still needs to be examined using nucleic acid-based methods of analysis. If such differences do exist in early life, our study suggests that they do not persist.

In addition to the differences in prevalence of bacterial species, it has been reported that differences exist in the composition of the *Bifidobacterium* population of allergic and non-allergic infants [34]. In particular, allergic infants were considered to have a more 'adult-like' microbiota because the majority were colonized with *Bifidobacterium adolescentis* (almost half exclusively with this species), compared with none of the non-allergic infants. Also, the majority of non-allergic infants were almost always colonized with *Bifidobacterium bifidum* (5/6) compared with only one of the allergic children. In our study we found no such differences, with the majority of both groups being colonized with *B. adolescentis* and approximately 40% of both groups being colonized with this species exclusively. In addition, very few children (approximately 15% of both groups) were colonized with *B. bifidum*. Once again, if differences between the composition of the *Bifidobacterium* population existed in early life, these differences do not persist.

Conclusions

We did not detect any difference in fecal microbiota composition between sensitized wheezy and non-sensitized, non-wheezy young children. It is possible that differences might have occurred in the fecal microbiota composition earlier in life and disappeared by the time our subjects were sampled. If this is the case then our results indicate that specific characteristics of the fecal microbiota are not the maintaining force of atopic wheeze. Alternatively, it may be that such differences only occur in allergic children with eczema as opposed to those with atopic respiratory disease. Further studies using nucleic acid-based techniques are necessary in order to establish whether differences between fecal microbiota composition truly exist in younger allergic and non-allergic children with different allergic diseases. Clarifying this issue may help in establishing the aetiology of the different clinical manifestations of allergic disease. From this study we predict that if the mechanism for the benefit of the use of probiotics in allergic disease is to modify intestinal microbiota composition, then using probiotics containing Lactic Acid bacteria and/or bifidobacteria in older children with wheeze would be unsuccessful. Further studies are required in order to ascertain whether probiotic use would be beneficial to wheezy atopic children.

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