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Intestinal microbiota and immunoglobulin E responses in 5-year-old Estonian children

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Summary

Background Over the last few decades, several studies from different parts of the world have indicated an increasing prevalence of allergic diseases. This has been related to environmental factors, like changes of microbial pressure. Our previous studies have demonstrated differences in the intestinal microbiota between allergic and non-allergic children.

Aim To test the hypothesis that the intestinal microbiota and IgE response are related, both in allergic and non-allergic 5-year-old Estonian children.

Methods The study group comprised 19 allergic and 19 non-allergic 5-year-old children, selected from a larger group who had been followed from birth. The diagnosis of allergy was based on clinical examination of the children and on data obtained from the questionnaires. The faecal microbiota were quantified by seeding serial dilutions on nine different media for incubation in different environment. The composition of the gut microbiota was expressed both as absolute counts of the various species and their relative share among the total counts of identified microbiota.

Results Bifidobacteria were less commonly detected in children with allergic diseases than in healthy children and clostridia comprised a higher proportion among their gut microbes. Children with specific IgE antibodies to defined allergens had higher counts of clostridia and the counts of clostridia correlated with the level of serum IgE, but only so in allergic children. In non-allergic children, the serum IgE levels showed a positive correlation with the counts of bacteroides.

Conclusion The development of allergic diseases seems to be associated with the composition of the gut microbial ecosystem. High counts of potential pathogens, such as clostridia, are associated with clinical manifestations of allergy and IgE antibody formation.

Keywords allergy, bacteroides, bifidobacteria, children, clostridia, gut microbiota, IgE
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Introduction

Over the last few decades, several studies from different parts of the world have shown an increasing prevalence of allergic diseases [1, 2]. This has been related to environmental factors, such as changes in diet, altered living conditions and a decrease in the extent of exposure to infectious agents, including low exposure to microbial products in early childhood [2–5]. Other studies have shown that living on a farm is associated with a low prevalence of atopic disease, possibly because of a high exposure to microbial products [6–9].

The gastrointestinal tract is the largest reservoir of microorganisms [10, 11], also containing up to 70–80% of all immunoglobulin-producing cells [12]. The normal microbiota of the gastrointestinal tract are a major stimulus for the postnatal maturation of T cell function [13, 14]. Cell wall components of intestinal microbiota stimulate the production of different cytokines and may enhance immune responses [15–17]. Recognition of these signals is mediated by a series of receptors, e.g. Toll-like (TLR) and CD14 receptors, which are expressed on cells of the innate immune system [18]. It has been postulated that a high endotoxin level associated with CD14 and TLR-4 during early life may stimulate T-helper type 1 (Th1) maturation, and thus protect against the development of atopic disease [19]. A recent study, however, suggested that endotoxin may not in itself be a key factor for development of allergy. They claim microbial metabolic products, i.e. fatty acid [20, 21].

Previous studies have demonstrated differences in the composition of the intestinal microbiota between allergic and non-allergic children in early childhood [20, 22–24]. In a prospective study, performed in Estonia, with a low, and Sweden with a high prevalence of allergic disease, differences were shown in the composition of intestinal microbiota already during the first month of life between infants who developed or did not develop allergic manifestations [23]. Cross-sectional studies have shown differences between allergic and non-allergic children at 1 [20] and 2 years [22].

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We aimed to study whether the differences are also present in older children and to test the hypothesis that the intestinal microbiota and IgE response are interconnected.

Material and methods

Study groups

The study group comprised 38 Estonian 5-year-old children (17 boys and 21 girls). They were selected from a larger group, followed for the development of immune responses to allergens and allergic diseases from birth. The selection was based on the presence or absence of clinical allergic symptoms at 5 years. Eleven of the 19 allergic children had atopic dermatitis (AD), seven had bronchial asthma (BA) and five had allergic rhinitis (Table 1). Four of the children had two allergic manifestations. All the allergic children were either skin prick tests (SPTs) positive and/or had circulating IgE antibodies against at least one allergen. The 19 non-allergic children were all SPT negative and had no signs of clinical allergy at any time during the first 5 years of life, although three of them had low levels of allergen-specific circulating IgE.

The study design has been described in detail previously [25]. Briefly, the children were vaginally delivered at term and the perinatal period was uncomplicated. Follow-up was performed at 6 and 12 months and at 2 and 5 years of life. The diagnosis of allergy was based on clinical examination at 5 years and on the data obtained from the questionnaires. Circulating IgE antibodies were determined against two food allergens (egg white and cow's milk) and five inhalants (house dust mite (HDM), cat, dog, birch and timothy), using UniCAP (Pharmacia Diagnostics, Uppsala, Sweden). For the SPT, fresh egg white and cow's milk, and extracts of HDM, cat, dog, birch, timothy and mugworth were used (ALK Abelló, Denmark).

Ethical aspects

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

Bacteriological analyses

Stool samples (1–2 g) were collected at home and kept in the domestic refrigerator at 4 °C for not more than 2 h before transportation to the laboratory, where they were frozen at − 70 °C until analysis.

Weighed samples of faeces were serially diluted (10^{-2}–10^{-9}) in pre-reduced phosphate buffer (pH 7.2) in an anaerobic glove box (Sheldon Manufacturing Inc., Tartu, Estonia, with a gas mixture: 5% CO, 5% H_2, 90% N_2). A quantitative analysis of the gut micro-organisms was performed using seeding serial dilutions on nine freshly prepared media.

Yeast extract agar was used for total aerobe counts, yeast extract agar with 6.5% of sodium chloride for staphylococci, Endo agar for enterobacteria, de Man–Rogosa–Sharpe agar

Table 1. Diagnosis and atopic sensitization of allergic and non-allergic children at 5 years of age

Allergic children ($n = 19$)				Non-allergic children ($n = 19$)			
Diagnosis of allergic diseases	Positive SPT	IgE antibodies	Total IgE (kU/L)	Diagnosis of allergic diseases	Positive SPT	IgE antibodies	Total IgE (kU/L)
AD		Cat*	2	Negative		CM, EW, dog, HDM, birch, timothy	10
AD	EW*	Cat*	51	Negative		CM, EW, cat, dog, HDM, birch, timothy	13
AD	Cat*, Dog*		23	Negative		Dog	278
AD		EW	77	Negative			96
AD		CM, EW, HDM, birch, timothy	24	Negative			2
AD		HDM, timothy	515	Negative			2
AD	Timothy	timothy	36	Negative			3
AD	Cat, birch	birch	45	Negative			6
AR		CM, HDM, cat	8	Negative			6
AR		dog	18	Negative			89
BA	Cat, dog	cat	32	Negative			9
BA	HDM	EW, HDM, birch	1974	Negative			52
BA		timothy	113	Negative			43
BA		CM	158	Negative			27
BA		CM, EW, HDM, birch	121	Negative			76
AD, AR	Dog	EW, dog, timothy	73	Negative			18
AD, AR	Cat, dog, birch	cat, dog,	963	Negative			41
AD, BA	HDM*	ND	ND	Negative			32
AR, BA		CM, EW	26	Negative			8

AD, atopic dermatitis; AR, allergic rhinitis; BA, bronchial asthma; SPT, skin prick test; CM, cow's milk; EW, egg white; HDM, house dust mite; ND, not detected
*Positive test result only at 2 years of age.

(MRS; Oxoid) for microaerophiles as lactobacilli and streptococci, Wilkins–Chalgren agar (Oxoid) for total anaerobes, Wilkins–Chalgren agar with vancomycin and nalidixic acid supplement (Oxoid) for Gram-negative anaerobes as bacteroides, Wilkins–Chalgren agar with colistin and nalidixic acid supplement for Gram-positive anaerobes as bifidobacteria and eubacteria, Cefoxitin–Cycloserine–Fructose agar (Oxoid) with egg yolk and sodium taurocholate for *Clostridium difficile* and Sabouraud dextrose agar with penicillin (50 000 U/L) and streptomycin (40 000 U/L) for yeasts and fungi. The total counts of spore-forming clostridia were estimated on Wilkins–Chalgren agar after ethanol treatment [26]. The yeast extract agar, salt-yeast-extract agar, Endo and Sabouraud agar were incubated aerobically by 37 °C and inspected after 24 and 48 h. The MRS medium was incubated in a microaerobic atmosphere (incubator IG 150, Jouan, Tarto, Estonia, France, with a gas mixture: 10% CO_2) for 72 h. Different media for anaerobes were incubated in an anaerobic glove box for 5–6 days.

The colony counts of the different faecal dilutions were recorded and from the highest dilutions with growth all colonies of different morphology were analysed for identification. The micro-organisms were identified mostly on genus (coagulase-negative staphylococci, lactobacilli, streptococci, enterococci, enterobacteria, candida, bifidobacteria, bacteroides, eubacteria, Gram-positive anaerobic cocci and clostridia) and species (*Staphylococcus aureus, C. difficile*) level.

Standard methods were used for identification of enterobacteria and other Gram-negative bacteria [27]. A coagulase test (Oxoid) was used for differentiation of *S. aureus* and coagulase-negative staphylococci. Streptococci and enterococci were identified by absence of catalase production and differentiated by fermentation of esculine. Lactobacilli were identified after growth in selective MRS media, Gram-positive rod-shaped morphology and negative catalase test.

Anaerobes were identified by growth on selective media, colonial and cellular morphology and Gram-stain reaction after verifying their inability to grow in an aerobic and microaerobic environment. We identified anaerobic Gram-negative rods as bacteroides, Gram-positive rods as eubacteria, bifidobacteria and Gram-positive anaerobic cocci. *C. difficile* was identified by the ability to grow on CCFA, the colony and cellular morphology, positive Gram staining and typical smell. The other spore-forming clostridia were identified by ability to grow in an anaerobic milieu after ethanol treatment.

After the identification of micro-organisms growing as single colonies in the highest dilutions, the composition of the gut microbiota was expressed both as absolute counts of the various species and their relative share of the total isolated microbiota. The number of the various species was given as colony-forming unit per gram faeces (CFU/g) and expressed in $\log_{10}$. The detection level was $\geqslant 3 \log$ CFU/g. For each child the counts of different identified bacterial groups were calculated, which were summarized to obtain the total count of cultivable intestinal bacteria. The relative share of each bacterial group was calculated from total counts. The relative share of the various bacteria as a percentage of the total counts of all isolated micro-organisms helps to estimate the balance of the intestinal microbial ecosystem. The prevalence (%), counts (log; CFU/g) and proportion (%) of the various bacteria were compared between different groups of children.

Statistical analysis

The statistical analyses were performed using 'SigmaStat' (Jandel Scientific, Tarto, Estonia, address USA) and 'Excel' (Microsoft Corp.) software programs, using the Fisher test, Spearman rank order correlation and Mann–Whitney *U* rank sum test. *P*-values less than 0.05 were considered to be statistically significant.

Results

Bifidobacteria were less commonly detected in allergic than in non-allergic children (1/19 vs. 7/19; $P = 0.04$, Fisher's test; Table 2). Thus, none of the children with AD and only one child with BA was colonized with bifidobacteria. The counts of the other components of the gut microbiota were similar in the allergic and non-allergic children (Table 2). The relative share of clostridia was higher, however, and that of bifidobacteria lower in allergic than in non-allergic children, i.e. mean proportion 12 vs. 3.9% for clostridia ($P = 0.03$, Mann–Whitney test) and 0.9 vs. 7.9% for bifidobacteria ($P = 0.04$, Mann–Whitney test; Fig. 1).

Furthermore, the counts of clostridia were significantly higher in children with than without specific IgE antibodies to at least one allergen (median 9.2, range 4.1–10.3 vs. median 6.9, range 3.8–9.9; $P = 0.02$, Mann–Whitney test; Fig. 2) at 5 years of life.

The levels of total serum IgE correlated with the bacteroide counts in non-allergic ($r = 0.577$; $P = 0.01$), but not in allergic children ($r = 0.284$; $P = 0.248$). Furthermore, the total serum IgE levels correlated with the counts of intestinal clostridia in allergic ($r = 0.550$; $P = 0.022$), but not in non-allergic children ($r = 0.432$; $P = 0.08$).

Table 2. Faecal microbiota in allergic and non-allergic 5-year-old children.

	Allergic children ($n = 19$)			Non-allergic children ($n = 19$)		
		Counts			Counts	
Micro-organisms	No. of children	Median	Range	No. of children	Median	Range
Aerobes	19	8.9	4.8–10.7	19	9.2	5.3–10.3
Anaerobes	19	10.5	8.4–11.2	19	10.6	8.6–11.2
CONS	9	6.6	3.3–8.8	10	7.6	3.8–10.2
S. aureus	7	5.2	3.3–7.3	4	4.4	3.6–8.3
Enterococci	13	6.3	3.6–9.9	14	7.3	3.3–9.9
Enterobacteria	18	7.3	4.3–9.3	17	7.6	4.3–9.6
Streptococci	10	7.7	4.3–10.6	11	8.7	4.3–10.1
Lactobacilli	11	7.8	3.3–10.4	10	5.5	4.3–9.3
Candida	5	5.3	3.3–5.9	6	5.3	4.3–7.3
GPAC	18	9.9	8.3–10.4	19	9.6	8.3–10.8
Bifidobacteria	1*	9.3	9.3	7*	9.3	6.3–10.6
Eubacteria	9	9.3	7.8–10.3	8	9.0	7.3–9.6
Bacteroides	19	10.3	7.8–11.1	19	10.0	7.3–10.9
Clostridia	18	9.3	4.1–10.3	17	7.6	3.8–10.3

*$P = 0.04$

CONS, coagulase negative staphylococci; *S. aureus*, *Staphylococcus aureus*; GPAC, Gram-positive anaerobic cocci.

The table shows the prevalence of colonization (number of children) and counts (log; CFU/g, range and median).

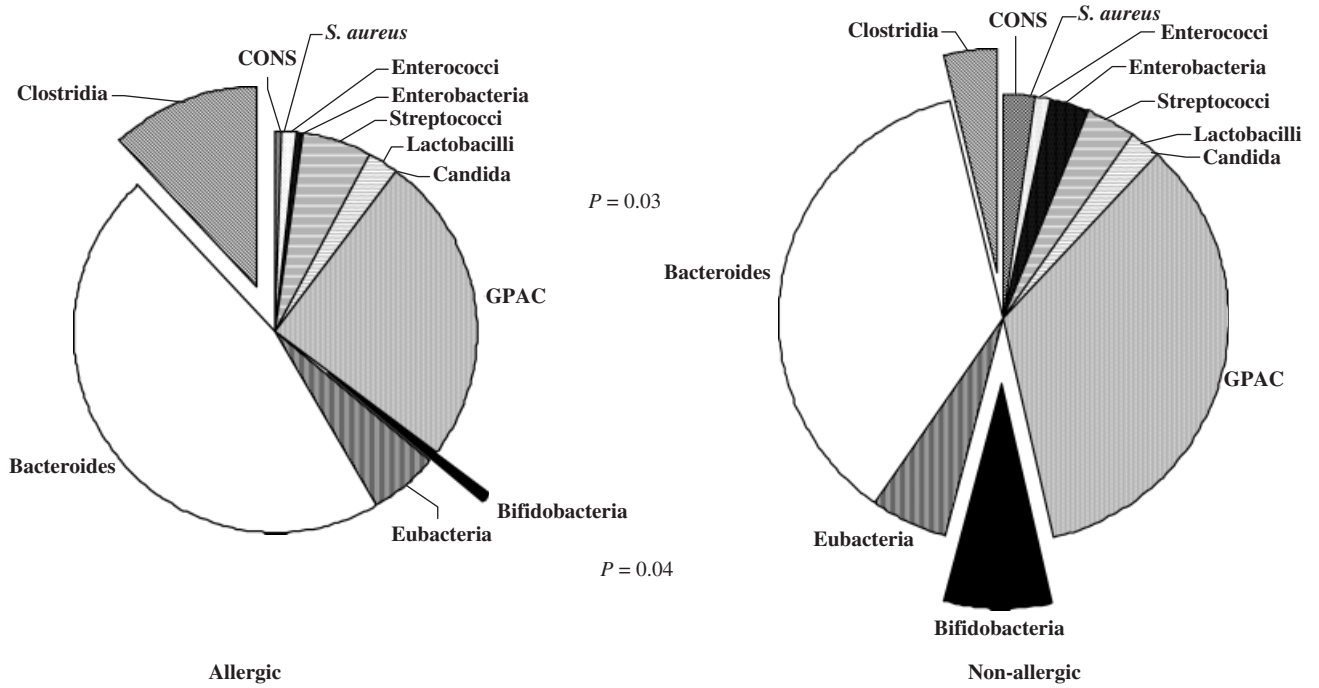

Fig. 1. Relative share (%) of the intestinal microbiota in 19 allergic and 19 non-allergic children at 5 years of age.

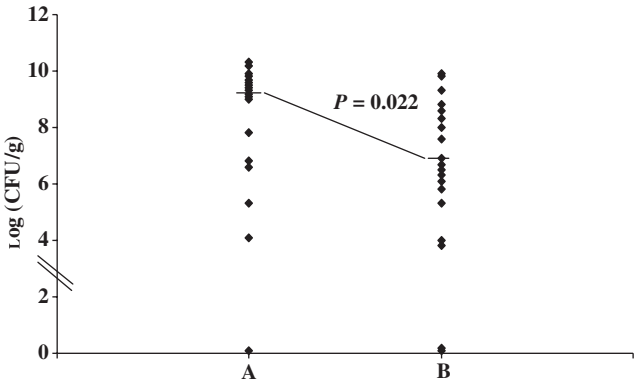

Fig. 2. Counts of clostridia (log; CFU/g) in children with (*A*; $n = 18$) and without (*B*; $n = 19$) specific IgE antibodies to foods and inhalant allergens.

Discussion

Using bacteriological culture methods, bifidobacteria were less commonly detected in children with allergic diseases than in non-allergic children and clostridia comprised a higher proportion of those gut microbes that were isolated. These findings confirm and extend previous observations in infants [23, 24] and adults with AD [28]. In addition, we showed that high counts of clostridia were present in children with IgE antibodies against one or more allergens and these correlated with total serum IgE levels in allergic children. These findings confirm that clostridia are associated with childhood allergy, as has been observed in infants at 3 weeks [24] and at 1 year [20].

There are several possible explanations for the relationship between the presence of clostridia in the gut microbiota and allergic manifestations. First, clostridia suppress a Th1 response in an animal model of inflammatory bowel disease [14]. A second mechanism could be through breaking oral tolerance [12], as clostridia may cause inflammation in gut tissues, leading to increased permeability of the mucosal barrier and thus facilitating the penetration of allergens [29, 30]. It is also possible that the increase of clostridia may be reflecting the shifts of some other, unknown components of the gut microbiota, as has been previously suggested for children from countries with high prevalence of allergies [21, 30, 31]. However, the presence of clostridia in individuals with specific IgE antibodies living in a country with a low prevalence of allergy like Estonia suggests more likely a causal relationship.

The development of allergic diseases seems to be associated with an imbalance of the gut microbial ecosystem where normally the colonization resistance-granting major groups of anaerobes as bifidobacteria, eubacteria, bacteroides and Gram-positive anaerobic cocci suppress the potentially pathogenic micro-organisms such as aerobes and clostridia [32–34]. During the first year of life, bifidobacteria are of particular significance [35, 36]. In the present study, cultivable bifidobacteria showed a lower proportion in the faecal microbiota of 5-year-old allergic children as compared with healthy ones. However, it is possible that the allergic and non-allergic children were colonized by different species of bifidobacteria with a varying ability to grow *in vitro* as it has been reported that some species of bifidobacteria can be recognized only by molecular methods [37].

The inhibitory effect of bifidobacteria on clostridia is associated with the production of short-chain fatty acids [38]. Furthermore, an inverse relationship has been shown in gnotobiotic mice between the presence of bifidobacteria and clostridia in the intestinal tract [38]. Administration of bifidobacteria enhanced antigen-specific IgA antibody pro-

duction against oral pathogens and protected against infections in mucosal tissues [39]. Increased counts of gut clostridia have been reported in humans with IgA deficiency [40].

Bacteroides become one of the major components of the cultivable gut microbiota from the second year of life [35, 36]. This was not the case, however, in our previous study of 2-year-old allergic children [22]. The present study extends our previous observations, showing a positive correlation between total serum IgE levels and bacteroide counts in non-allergic children at 5 years. A similar tendency towards lower counts of faecal anaerobic bacteria was recently reported in atopic adults [41]. Possibly, high numbers of bacteroides may provide an anti-inflammatory stimulus, which protects the child against allergic manifestations, despite elevated serum IgE. Several studies hint that bacteroides promote isotype switch to IgA in B cells and induces oral tolerance via the activation of an antigen-specific non-response to an antigen [14]. Recently the induction of transforming growth factor (TGF)-β by Th3 regulatory cells was shown by *Bacteroides vulgatus*, a member of gut microbiota [42].

In conclusion, high counts of clostridia are associated with manifestation of allergy and specific IgE responses, while bifidobacteria are inversely related to allergic disease, at least up to 5 years of age. Bacteroides seems to be associated with down-regulation of allergic response in non-allergic children with high serum IgE level. Thus, a balanced composition of gut-cultivable microbiota, seemingly because of the presence of anaerobes, such as bifidobacteria and bacteroides, may protect children against allergic diseases, not only in infancy but also later in childhood. Human microbial ecology is still poorly understood and therefore the presence or absence of a single species should be interpreted with caution. This and several previous studies do, however, strongly suggest a relationship between childhood allergy and disturbed gut microbiota.

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