

# The intestinal microflora in allergic Estonian and Swedish 2-year-old children

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## Summary

**Background** The prevalence of allergic diseases seems to have increased particularly over the past 35–40 years. Furthermore, allergic disease is less common among children in the formerly socialist countries of central and Eastern Europe as compared with Western Europe. It has been suggested that a reduced microbial stimulation during infancy and early childhood would result in a slower postnatal maturation of the immune system and development of an optimal balance between TH1- and TH2-like immunity.

**Aims** To test the hypothesis that allergic disease among children may be associated with differences in their intestinal microflora in two countries with a low (Estonia) and a high (Sweden) prevalence of allergy.

**Methods** From a prospective study of the development of allergy in relation to environmental factors, 29 Estonian and 33 Swedish 2-year-old children were selected. They were either nonallergic ( $n = 36$ ) or had a confirmed diagnosis of allergy ( $n = 27$ ) as verified by typical history and at least one positive skin prick test to egg or cow's milk. Weighed samples of faeces were serially diluted ( $10^{-2}$ – $10^{-9}$ ) and grown under anaerobic conditions. The counts of the various genera and species were calculated for each child. In addition, the relative amounts of the particular microbes were expressed as a proportion of the total count.

**Results** The allergic children in Estonia and Sweden were less often colonized with lactobacilli ( $P < 0.01$ ), as compared with the nonallergic children in the two countries. In contrast, the allergic children harboured higher counts of aerobic micro-organisms ( $P < 0.05$ ), particularly coliforms ( $P < 0.01$ ) and *Staphylococcus aureus* ( $P < 0.05$ ). The proportions of aerobic bacteria of the intestinal flora were also higher in the allergic children ( $P < 0.05$ ), while the opposite was true for anaerobes ( $P < 0.05$ ). Similarly, in the allergic children the proportions of coliforms were higher ( $P < 0.05$ ) and bacteroides lower ( $P < 0.05$ ) than in the nonallergic children.

**Conclusions** Differences in the indigenous intestinal flora might affect the development and priming of the immune system in early childhood, similar to what has been shown in rodents. The role of intestinal microflora in relation to the development of infant immunity and the possible consequences for allergic diseases later in life requires further study, particularly as it would be readily available for intervention as a means for primary prevention of allergy by the administration of probiotic bacteria.

**Keywords:** Intestinal microflora, allergy, children, food, *Lactobacillus*, *Clostridium difficile*

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## Introduction

There is considerable evidence that the prevalence of atopic

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diseases is increasing in industrialized countries with a market economy [1]. In contrast, the prevalence of allergic disease is much lower among children and young adults in the formerly socialist countries of central and Eastern Europe, e.g. Estonia [2], former German Democratic Republic [3] and Poland [4]. In

the beginning of the 1990s the lifestyle of populations in these countries were in several ways similar to that prevailing in Western Europe some 30 years earlier.

The reasons for the differences in allergy prevalence between Eastern and Western Europe are unknown. It has been suggested that the increasing prevalence of allergic diseases in Western Europe is due to less microbial pressure in early childhood as a consequence of improved hygienic conditions [5–7]. An inverse relationship between the presence of allergic disease and tuberculin reactivity has also been reported [8]. These observations may be spurious, however, since it has been known for a long time that atopic individuals manifest reduced delayed-type hypersensitivity reactions as compared with nonatopic individuals [9]. Furthermore, occasional respiratory tract infections, even if appearing fairly often, would not be expected to exert a continuous pressure on the immune system as to induce an immune deviation similar to what has been shown in animal experiments [6].

It has been known for decades that gut commensal microbes, colonizing the neonatal mammal, affect not only the development of oral tolerance but also the development of the systemic immune system, continuously exerting a pressure on the immune system [10–13]. The microbial flora is relatively stable, once it has been established in infancy, provided there are no major changes in the diet [14–16]. It is therefore tempting to suggest that the gut's indigenous flora could exert such continuous stimulation of the immune system as to enhance immune deviation. However, very little is known regarding the possible immune modulating effects of each of the numerous different, mostly non-characterized bacterial species that are present in the gut.

There are large geographical variations in the composition of the human gastrointestinal microflora [17,18]. We have recently observed considerable differences in the composition of the intestinal flora in Estonian and Swedish one-year-old infants [19]. The differences included a more intensive colonization with lactobacilli and eubacteria in the former children, whereas the Swedish infants had increased numbers of clostridia, particularly *C. difficile*, as well as bacteroides and other anaerobes.

We have tested the hypothesis that allergic disease among children may be associated with differences in their intestinal microflora, by analysing the composition of intestinal microflora in two different populations, i.e. Estonia with a low and Sweden with a high prevalence of allergy [4] at the age of two years.

## Materials and methods

### Study groups

The study groups comprised 29 Estonian and 33 Swedish

children aged two years, with equal numbers of boys and girls. They all participated in a prospective study of allergic disease in relation to environmental factors starting at birth. Clinical follow-up focusing on a history and signs of allergic symptoms were performed at 6 and 12 months, and at 2 years. On these occasions, venous blood samples were obtained and skin prick tests performed with egg and cow's milk, as well as with cat, dog, timothy and birch pollen extracts (ALK, Hørsholm, Denmark) at 2 years. The duration of exclusive or partial breast feeding and the number of infants treated with antibiotics were similar in the two groups. The children were selected from a larger cohort based on a convincing history of either the presence or absence of allergic disease up to 2 years of age. In all, 27 children with confirmed allergic disease (13 and 14 children, respectively, in the two countries), based on atopic dermatitis as suggested by Hanifin and Rajka [20] and at least one positive skin prick test during the first 2 years of life were included. None of the 35 nonallergic children (16 and 19 in Estonia and Sweden, respectively) had a positive skin prick test at any time up to two years of age and none of them had any history of eczema, nor wheezing.

Faecal samples were collected at home. Approximately one gram voided stool was collected into sterile plastic containers by the parents and frozen at  $-20^{\circ}\text{C}$  within two hours.

The study was approved by the Institutional Review Boards at Linköping and Tartu universities.

### Bacteriological analysis

Weighed samples of faeces were serially diluted ( $10^{-2}$ – $10^{-9}$ ) under a stream of  $\text{CO}_2$  in prerduced phosphate buffer (pH 7.2). The bacteria were quantified by serial dilutions (0.05 mL) on 10 freshly prepared media as recently described in detail [16]. The microbial counts were given in log colony forming units per gram faeces (CFU/g). The detection limit of microorganisms was 3.0 log CFU/g. The microorganisms were identified mostly on genus level. For identification of enterobacteria on species level standard methods were used.

### Statistical methods

The counts (log 10 CFU/g) of the various genera and species were calculated for each child. In addition, the relative amounts of the particular microbes were expressed as a proportion of the total count. Since the counts were not normally distributed, nonparametric tests (Mann–Whitney Rank Sum test and Kolmogorov–Smirnov two-sample test) were used to compare colonization prevalence and counts in allergic and nonallergic Estonian and Swedish children. As the relative proportions of microorganisms in the children

were normally distributed after log-transformation, they were compared employing student's *t*-test.

## Results

The allergic children were less often colonized with lactobacilli and bifidobacteria, as compared with the nonallergic children (Table 1). The trends were similar for both the Estonian and Swedish children, although statistically significant only for lactobacilli (Figs 1 and 2). In contrast, the allergic children harboured higher counts of aerobic microorganisms, particularly coliforms in the Estonian and *S. aureus* in the Swedish children. The prevalence and the counts of enterococci, streptococci, candida, clostridia, *C. difficile*, peptostreptococci, eubacteria and bacteroides were similar in the allergic and nonallergic children in both countries (Table 1). Only for coagulase-negative staphylococci did the trends differ between allergic and nonallergic children in the two countries (Figs 1 and 2).

The numbers of aerobes, expressed as a proportion of the total intestinal microflora, was also higher in allergic as compared with nonallergic children, while the proportion of anaerobes was lower (Fig. 3). Similarly, the proportion of enterobacteria was higher and bacteroides lower in allergic, as compared with nonallergic children. The trends were similar in the Estonian and Swedish children (data not shown).

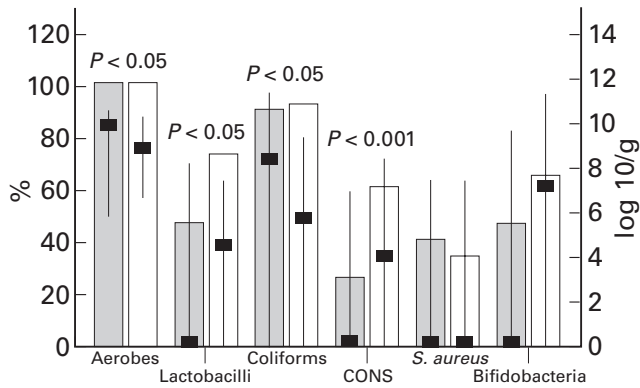
## Discussion

The intestinal microflora differed in 2-year-old allergic and nonallergic children, both in Estonia and Sweden, including fewer microaerophilic lactobacilli, bifidobacteria and bacteroides and more aerobic microorganisms such as coliforms and *S. aureus*. We could not exclude other differences between the study groups as the groups were small. Furthermore, there is a risk for mass significance when multiple comparisons are performed comparing the prevalence and counts of numerous bacterial species in two countries, as in this study. The differences between allergic and nonallergic children were largely similar in Estonian and Swedish children, however. The study also confirmed our recent observations in one-year-old infants that a lactic acid microflora, including lactic acid-producing streptococci, is more common in the Estonian children [16]. The low counts of lactobacilli in many Swedish children support previous findings of high counts of lactobacilli in the fecal flora of newborn infants in a developing country (Ethiopia) to be more common than in Sweden [15].

The microflora in the allergic children was similar to that previously described as an imbalance in the microflora of adults characterized by the suppression of symbiotic anaerobes and an overgrowth by some potentially pathogenic microorganisms as a consequence of antimicrobial treatment [21]. However, according to the questionnaires completed by the parents, the number and type of antibiotic

**Table 1.** Composition of the faecal microflora in allergic and nonallergic 2-year-old Estonian and Swedish children. The table shows the prevalence of various species (%) and the counts (log CFU/g). The median values are given for positive samples. The level of significance for differences in prevalence and counts in positive samples between the two groups are indicated by \* –  $P < 0.05$  and \*\* –  $P < 0.01$ .

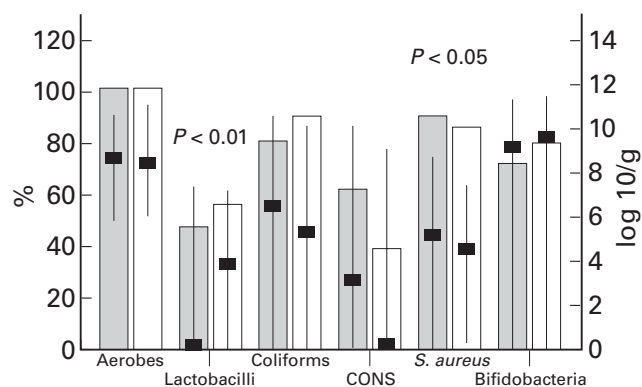
Microbes	Allergic, <i>n</i> = 27			Nonallergic, <i>n</i> = 35		
	%	Median	Range	%	Median	Range
Aerobes	100	9.3*	4.8–11.1	100	8.3*	6.0–11.1
Lactobacilli	44**	4.8	– 8.3	60**	5.3	– 7.8
Coliforms	85	8.3**	– 10.9	94	6.3**	– 10.6
CONS	41	5.3	10.3	46	5.8	9.3
<i>S. aureus</i>	63	5.6*	– 8.3	60	5.1*	– 7.6
Enterococci	85	8.1	– 10.8	89	7.7	– 11.3
Streptococci	30	6.7	– 10.6	29	7.9	– 9.8
<i>Candida</i>	30	3.8	– 5.3	37	4.3	– 6.6
Anaerobes	100	9.6	5.0–11.3	100	9.9	5.1–11.8
Bifidobacteria	59*	9.4	– 11.1	71*	9.3	– 11.3
Clostridia	67	7.4	– 11.3	77	7.3	– 10.6
<i>C. difficile</i>	15	5.8	– 7.6	23	5.5	– 7.9
Peptostrepto-cocci	67	9.1	– 10.6	69	8.3	– 10.3
Eubacteria	7	0	– 9.6	0	0	0
Bacteroides	78	9.3	– 10.8	94	9.3	– 11.7



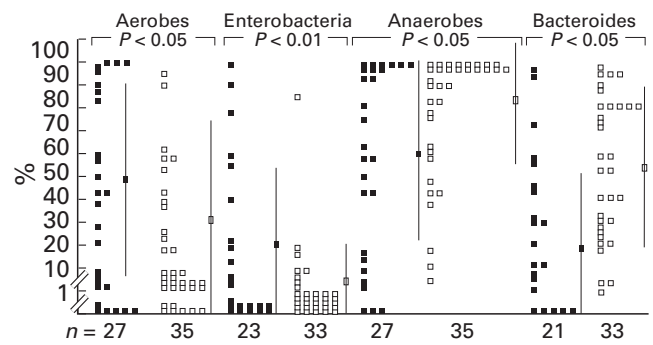
**Fig. 1.** Intestinal microflora of 13 allergic (grey) and 16 nonallergic (white) Estonian children. The results are presented as colonization rate (%), columns) and counts (log CFU/g, range and median, lines and filled symbols).

treatment between allergic and nonallergic children were similar.

A reduced microbial stimulation during infancy and early childhood has been associated with the increasing prevalence of asthma and allergy in children and young adults of developed countries [7]. Thus, less microbial pressure would result in a slower postnatal maturation of the immune system and thus a delayed development of an optimal balance between TH1- and TH2-like immunity [6]. It is not likely, however, that viral and bacterial infections in the respiratory tract, even if occurring fairly frequently, would exert such continuous pressure on the immune system as to accommodate the experimental observations in rodents. For these reasons the intestinal microflora would be a more likely source of stimulation during the maturation of immunity. In germ-free newborn rodents tolerance induction is delayed and it can be restored by



**Fig. 2.** Intestinal microflora of 14 allergic (grey) and 19 nonallergic (white) Swedish children. The results are presented as colonization rate (%), columns) and counts (log CFU/g, range and median, lines and filled symbols).



**Fig. 3.** Relative amounts of aerobic and anaerobic microorganisms, enterobacteria and bacteroides, expressed as a percentage of the total microbial faecal flora in allergic ( $n = 27$ , filled symbols) and nonallergic ( $n = 35$ , open squares) 2-year old children.

neonatal colonization with normal commensals of the intestinal flora [22]. It is conceivable that the indigenous intestinal flora might affect the development and priming of the immune system in early childhood, similar to what has been shown in rodents.

Virtually nothing is known regarding what bacterial species might be involved in this process. Lactobacilli may be of particular interest in this respect, however, as at least some strains can induce the synthesis of interferon- $\gamma$  [12] which is involved in the downregulation of TH2-type cytokines [23]. They have also been suggested to generate tolerogenic peptides from native protein and to promote intestinal barrier mechanisms [24]. Lactobacilli are regarded as harmless bacteria and over the past few years have generated an increasing interest as potentially probiotic bacteria [24]. Very recently, administration of a strain of *Lactobacillus*, was associated with a reduced severity of atopic dermatitis in infants in a double-blind, placebo-controlled study over one month [25]. The high prevalence of lactobacilli among young children in Estonia with a low prevalence of allergic disease [16] is of interest in this respect.

A disturbed vaginal microflora of their mothers or too strict hygiene during birth-giving have been offered as a possible explanation for the low counts of lactobacilli in Sweden [26]. Alternatively, increased consumption of mostly industrially processed and sterilized foods, as major components of the diet in western industrialized countries may influence the intestinal flora, causing a reduction in lactic acid producing microbes [27].

The prevalence of allergic diseases seems to have increased particularly over the past 35–40 years and several studies indicate that the large differences in the prevalence of allergic diseases between Eastern and Western Europe are limited to age groups born after the late 1950s [28]. This is well before the major changes in building techniques

occurred as a consequence of the first energy crisis and coincides with a time period when major changes in the diet began to take place in Western Europe, e.g. freezers, industrially processed foods and microwave ovens became more common in the homes. The role of intestinal microflora in relation to the development of infant immunity and the possible consequences for allergic diseases later in life require further study.

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