

Microflora-associated characteristics in faeces from allergic and nonallergic infants

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Summary

Background The prevalence of allergic diseases has increased particularly over the past 30–40 years. A reduced microbial stimulation during infancy may result in a development of a disturbed balance between Th1- and Th2-like immunity. The gut flora is, quantitatively, the most important source for such stimulation.

Objective The aim of the study was to compare the gut microbial flora in 25 allergic and 47 nonallergic 13-month-old infants (range 11–18), through analysing microflora-associated biochemical markers in faeces.

Methods Microflora associated characteristics (MACs) were assessed by determining the concentrations of eight different short chain fatty acids and the conversion of cholesterol to coprostanol by gas chromatography. Faecal tryptic activity was analysed spectrophotometrically.

Results The allergic infants had lower levels of propionic, i-butyric, butyric, i-valeric and valeric acid. In contrast, they had higher levels of the rarely detected i-caproic acid, which has been associated with the presence of *Clostridium difficile*. Furthermore, the allergic infants had higher relative distribution of acetic and i-caproic acid. None of the other parameters differed between the groups.

Conclusion The results demonstrate differences in the MACs between allergic and nonallergic infants, indicating differences in the composition of the gut flora that may disturb the development of a normal Th1-/Th2-balance in allergic children.

Keywords: allergy, infant, microflora-associated characteristic, intestinal microflora, short chain fatty acids

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Introduction

Immune responses to allergens are characterized by a cross-regulation between competing IFN γ -secreting T helper 1 (Th1)-like cell populations [1] and IL-4-producing Th2-like cell populations [2]. Atopic individuals display a bias towards the production of type 2 cytokines as compared to nonatopics, and allergic disease is associated with Th2-like immunity to allergens in mucosal tissues [2]. Primary T-cell responses to environmental allergens occur during a

period of immaturity of the immune system, i.e. during infancy or even before birth [3]. The fetomaternal interface is, during pregnancy, surrounded by high levels of the Th2-like cytokines [4], probably in order to divert the maternal immune response away from harmful Th1-mediated immune responses. This bias appears to be preserved for varying periods in infancy [5].

Microbial stimulation, both from normal commensals and pathogens, may be important for the postnatal development of a normal Th1-/Th2-balance. The increasing prevalence and severity of atopic diseases seen in industrialized countries [6,7] are in marked contrast with

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the low prevalence of allergy among children in the formerly socialist countries of Europe with a life style similar to that prevailing in Western Europe 30–40 years ago [8,9]. The increase may in part be caused by the major improvements in public health and personal hygiene practice, diet changes, reduction of average family size and improvements of general living standards, that is to say a changed overall exposure to microbial stimulation [10]. The gut flora is the quantitatively most important source of microbial stimulation and may provide a primary signal for driving the postnatal maturation of the immune system, thus inducing Th1-like immunity [10]. We have recently reported considerable differences in the composition of the gut flora of healthy 1-year-old infants in Estonia, with a low prevalence of allergy [8], and Sweden [11]. For example, *Lactobacilli* and *Eubacteria* are more common and the counts are higher in Estonian than in Swedish infants, whereas the reverse is true for *Clostridium difficile*. Very recently differences were confirmed in Estonian and Swedish 2-year-old children [12].

An alternative approach to study the microbial flora is to investigate the functional status of the flora, i.e. 'what have the microbes done?' A microflora-associated characteristic (MAC) has been defined as any anatomical, physiological, immunological or biochemical function in a macroorganism, which has been acted on by the microflora [13]. Examples of MACs include the formation of short chain fatty acids (SCFAs) by anaerobic microbes in the human colon, microbial transformation of cholesterol to coprostanol, and the microbial inactivation of intestinal trypsin which can be measured by the remained faecal tryptic activity (FTA) in faeces. In contrast to traditional isolation of specific microorganisms, the MACs are mainly used to monitor the establishment and the development of a normal gut flora in general, and not as markers for specific commensals.

This approach to study the gut flora has several advantages over more traditional analyses based on isolation and enumeration of the microorganisms, as the latter are expensive, time consuming and associated with problems in obtaining samples from parts of the GI-tract that are normally inaccessible.

The aim of this study was to test the hypothesis that differences in the maturation of the immune system in allergic and nonallergic infants could at least in part be a consequence of a different gut flora. This was done, employing MACs as indicators of the gut microbial status.

Materials and methods

Study group

Faecal samples were obtained from 72 infants participating

in a prospective study including all children that were born during 1 year in Östersund, Sweden. The infants in this study were selected on basis of the availability of faeces samples collected at 13 months. The infants were clinically examined at 12 (range 11–13) months of age. They were considered as allergic if they had at least one positive skin prick test (SPT) to at least one out of five allergens and allergic symptoms ($n = 25$) and as nonallergic if they had negative SPT and no allergic symptoms ($n = 47$). Information regarding the use of antibiotics and the length of exclusive breast feeding was also recorded. The children were exclusively breast-fed for a median time period of 4 (range 0–6) months, and 16 of the children had received at least one antibiotic treatment before the faecal samples were collected. None of the children received any antibiotic treatment within 3 months before the faeces sample was collected, however. None of the children had a confirmed food allergy and was therefore not avoiding any particular food.

Skin prick tests were done on the volar aspects of the forearms with standardized egg, milk, birch, timothy and cat extracts (Soluprick, ALK, Hørsholm, Denmark). Histamine hydrochloride (10 mg/mL) was used as a positive control.

Atopic dermatitis was defined as pruritic, chronic or chronically relapsing noninfectious dermatitis with typical features and distributions. Asthma was defined as three or more episodes of bronchial obstruction or at the first occasion of bronchial obstruction if the child had any other atopic symptoms. Vomiting and/or diarrhoea on at least two separate occasions after intake of the offending food was considered as gastrointestinal allergy.

The faecal samples were collected at the age of 13 months (range 11–18) at home by the parents in a plastic tube and immediately put in -20°C . The samples were delivered frozen to the hospital and stored at -20°C for 2–14 months until analysed. Before the analyses, the samples were thawed at room temperature.

Determination of SCFAs in faeces

Aliquots of faeces (0.4–0.6 g) were homogenized in 2 mL of distilled water containing 3 mmol/L of 2-ethylbutyric acid as an internal standard and 0.5 mL of H_2SO_4 (0.5 mmol/mL). The homogenate was vacuum distilled and analysed for SCFAs as described by Zijlstra *et al.* [14] with modifications by Høverstad *et al.* [15] on a gas chromatograph (Pye-Unicam GCD-104). Chromatograms were recorded, peak areas determined and concentrations calculated with the Shimadzu Data Processor Chromatopac C-R3A. Each series of analyses (10–15 samples) started and ended with the injection of a standard solution. From these standard chromatograms, the relative response factors

for that batch were obtained. The concentrations of the different SCFAs were expressed as mmol/kg faeces and as relative distribution for each SCFA out of the total amount of SCFAs from one sample. The detection level was 0.2 mmol/kg faeces.

Determination of conversion of cholesterol to coprostanol

Aliquots of the faeces (0.5–1.0 g) were mixed with 0.9% NaCl in sterile deionized water (1 : 2 w/v), placed at 4 °C for 2 h and then centrifuged for 30 min at 4 °C and 35 000 g. The supernatants were stored in aliquots at –20 °C until analysed for faecal tryptic activity (FTA). The sediment, used for determination of cholesterol conversion, was extracted and analysed as described by Midtvedt *et al.* [16] on a gas chromatograph (Hewlett Packard 5880 A Gas Chromatograph). The values were expressed as percent coprostanol out of total amount of cholesterol and coprostanol present. Peak areas < 1% were neglected as impurities.

Determination of faecal tryptic activity

The supernatants (see above) were thawed at room temperature and aliquots of 0.1 mL were added to 2.9 mL Tris buffer, pH 8.2. Then, 0.6 mL of the substrate, 0.003 M N-benzoyl-DL-arginine-4 nitroanilide hydrochloride (BAPNA, Sigma Aldrich, Stockholm, Sweden) was added. The reaction was performed at room temperature and stopped after 10 min by adding 0.6 mL 5 M acetic acid. Bovine pancreas trypsin type III (Sigma Aldrich, Stockholm, Sweden) diluted in 2 mM HCl was used for construction of a standard curve. All samples and standards were run in duplicate and analysed spectrophotometrically (HITACHI 150–20 Spectrophotometer) in parallel with blanks at 405 nm. After corrections for blank values, FTA was calculated and expressed as mg tryptic activity/kg faeces. The detection level was 3 mg/kg faeces.

Statistical methods

As the variables were not normally distributed even after logarithmic transformation, nonparametric tests, corrected for ties, were used. Comparisons between unpaired groups were analysed with Mann–Whitney *U*-test and correlations with Spearman's rank order correlation coefficient test. Chi-square test was used for categorical determinations. A probability level of < 5% was considered to be statistically significant. Calculations were performed with a statistical package StatView 4.5 for Macintosh (Abacus Concepts Inc, Berkeley, CA, USA).

Ethical aspects

The study was approved by the Regional Ethical Committee for Human Research at the University Hospital, Umeå, Sweden.

Results

Eight SCFAs were analysed in the samples, i.e. acetic, propionic, i-butyric, butyric, i-valeric, valeric, i-caproic and caproic acid. Acetic, propionic and butyric acid were detectable in all samples (Table 1). Lower levels of i-butyric, i-valeric and valeric acid were detected in most of the samples. Iso-caproic and caproic acid were detectable in only seven and six of the 72 samples, respectively, and then in low concentrations.

The nonallergic, as compared to the allergic infants had higher levels of propionic, i-butyric, butyric, i-valeric and valeric acid in their faeces (Fig. 1). Furthermore, the relative distribution of propionic and valeric acid was higher in faeces from nonallergic infants and similar trends were seen for i-butyric and i-valeric acid (Fig. 3).

In contrast, the levels of i-caproic acid (Fig. 2) and the relative distribution (Fig. 3) of i-caproic and acetic acid were higher in the allergic, as compared to the nonallergic, infants.

There were no associations between the levels of the SCFAs and the length of breast feeding, use of antibiotics

Table 1. Short chain fatty acids in faeces

<i>n</i> = 72	acet	prop	i-bit	but	i-val	val	i-cap	cap
Detected <i>n</i>	72	72	68	72	66	39	7	6
mmol/kg faeces median	63	17	1.0	14	1.1	0.2	< 0.2	< 0.2
Range	16–134	2–53	< 0.2–7	2–65	< 0.2–7.7	< 0.2–5.6	< 0.2–3.2	< 0.2–0.4
% of total median	63	16	1	14	1	<	<	<
Range	23–94	3–40	< –10	2–32	< –6	< –4	< –2	<

The median and range are given for the concentrations (mmol/kg faeces) and the relative distributions (% of the total amount SCFAs in one sample) of the different acids. The number of samples with detectable levels are those above the cut off (0.2 mmol/kg faeces). Acet = acetic, prop = propionic, I-but = iso-butyric, but = butyric, I-val = iso-valeric, val = valeric, I-cap = iso-caproic and cap = caproic.

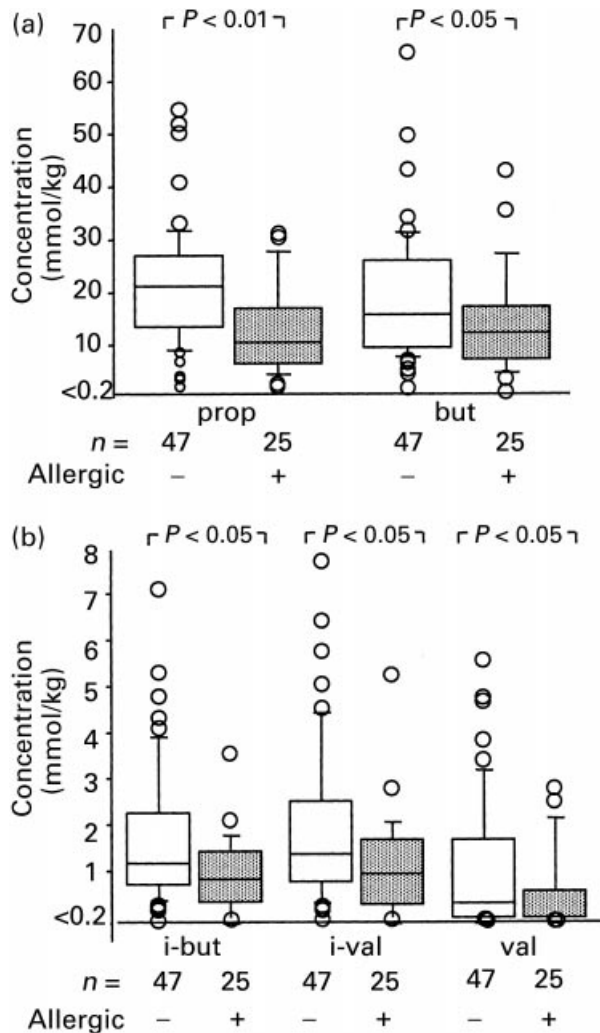


Fig. 1. Concentration (mmol/kg faeces) of propionic (prop) and butyric (but) acid (a) and iso-butyric (i-but), iso-valeric (i-val) and valeric (val) acid (b) in faeces from 25 allergic and 47 nonallergic infants. The detection level was 0.2 mmol/kg faeces. The 10th, 25th, 50th, 75th and 90th percentiles are indicated, as well as outliers.

or age, except for the concentration of propionic acid ($\rho = -0.32$, $P < 0.05$) and age. No such correlation with age was found within the allergic and nonallergic groups, however.

The time period of exclusive breast feeding and the number of antibiotic treatment were similar between the allergic and nonallergic children. The allergic infants, however, were younger than the nonallergic when the faeces samples were collected, i.e. median (range) were 13 (11–14) and 14 (12–18) months, respectively, $P < 0.001$.

The conversion of cholesterol to coprostanol and FTA were analysed in 46 of the 72 samples, 31 from nonallergic

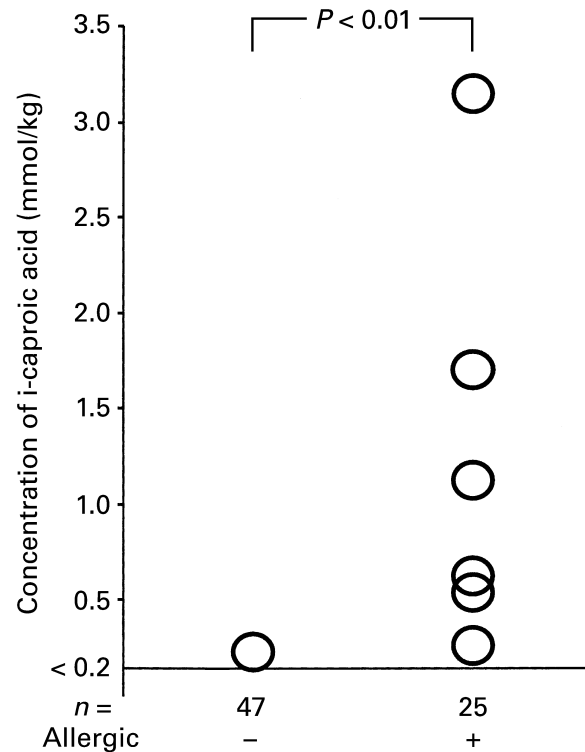


Fig. 2. Concentration (mmol/kg faeces) of i-caproic acid in faeces from 25 allergic and 47 nonallergic infants. The detection level was 0.2 mmol/kg faeces.

and 15 from allergic infants, while 26 samples could not be analysed due to too small volumes. Coprostanol was detected in 28 of the 46 samples (median = 3%, range: < 1–89%) and tryptic activity was detected in 44 of the 46 samples (median = 93 mg/kg faeces, range: < 3–590 mg/kg faeces). The cholesterol/coprostanol ratio and the FTA were similar in the allergic and nonallergic infants and there were no correlations to age, length of breast-feeding period or use of antibiotics.

Discussion

Interest has recently been focused on the protective role of early infections on the development of allergy [17], possibly through Th-2 inhibitory effects of cytokines released during host defence responses to infections. It may seem unlikely however, that a stimulus, that is potentially harmful to the host, should be necessary for the postnatal maturation of a balanced immune system. It has been suggested that the primary signal for such maturation during infancy and early childhood is provided not by pathogens, but by stimulation from the commensal microbial flora, where the flora of the gastrointestinal tract is the most quantitatively important [10]. Therefore, we

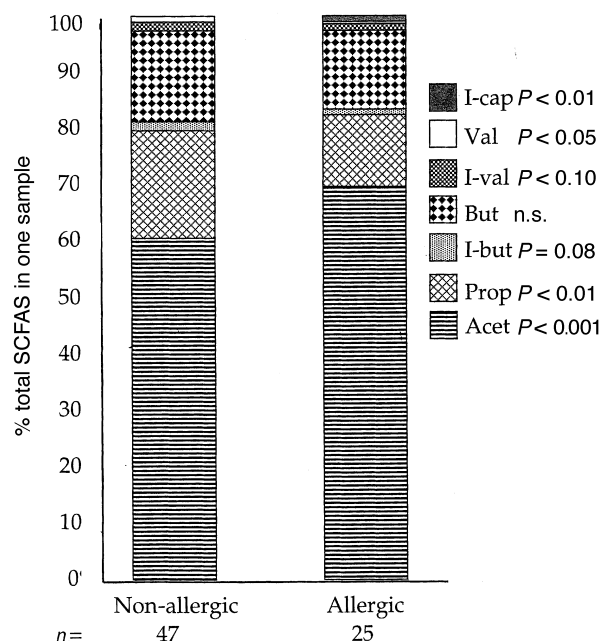


Fig. 3. The median relative distribution (% of the total amount SCFAs in one sample) of acetic (acet), propionic (prop), iso-butyric (i-but), butyric (but), iso-valeric (i-val), valeric (val) and iso-caproic (i-cap) acid in faeces from 25 allergic and 47 nonallergic infants. The allergic, as compared to nonallergic, children had higher relative distribution of iso-caproic and acetic acid. The other acids, except for butyric acid, were higher in the nonallergic children.

wanted to investigate if there were differences in the gut flora, monitored by MACs, between allergic and non-allergic children.

The allergic infants had higher levels and higher relative distribution of i-caproic acid in the faeces, as compared to nonallergic infants. This uncommonly detected acid is suggested to indicate the presence of *Clostridium difficile* in the gut [18]. There is evidence that a normal gut flora provides protection against a broad range of enteric pathogens [19], including *C. difficile* [20]. The higher levels of i-caproic acid among the allergic, as compared to nonallergic, infants may therefore indicate a disturbed gut flora with higher counts of *C. difficile* in the former group. In another study it was found that the gut flora of Estonian children, a population with a low prevalence of allergy [8], more often comprised *Lactobacilli* and *Eubacteria*, when compared to Swedish children, whereas the reverse was true for *C. difficile* [11]. Very recently differences were confirmed in Estonian and Swedish two year old children [12].

It has been shown that lactobacilli can prevent the colonization of *C. difficile* [21]. The lower frequency of lactobacilli among allergic infants may thereby be a risk factor for colonization with *C. difficile* and could possibly

explain the higher levels of i-caproic acid in the faeces of these infants.

Similar to i-caproic acid, the relative distribution of acetic acid was higher in faeces from allergic, than nonallergic, infants. In contrast, the concentration of propionic, butyric, i-butyric, i-valeric and valeric acid were higher in the nonallergic infants. Raised concentrations of propionic acid in faeces together with high counts of *Lactobacilli plantarum* have been observed after administration of oats fermented with *L. Plantarum* [22]. This may indicate a correlation between levels of propionic acid and number of lactobacilli. Thus, the lower levels of propionic acid in faeces from allergic, as compared to nonallergic, children may indicate lower counts of lactobacilli.

It has been suggested that certain strains of lactobacilli, e.g. *L. casei*, can inhibit allergen-induced IgE production by murine splenocytes, possibly through induction of IL-12 secretion by macrophages [23]. Thus, the intestinal flora may play a crucial role in generating Th2 cell populations fully susceptible to oral tolerance induction [24]. Furthermore, the severity of atopic eczema improved in infants treated with extensively hydrolysed whey formula fortified with *Lactobacillico GG* [25].

Iso-butyric, i-valeric, valeric and caproic acid have not been correlated to any specific species of bacteria as they are produced by numerous strains throughout the colon. It has been suggested however, that the establishment of a normal gut flora can be monitored by the presence and amounts of the different acids [26]. In new born children, who are colonized with a limited number of bacterial species, acetic acid is the only SCFA produced. At 2 years, the production of the other SCFAs and an adult-like gut flora has been established. The lower levels of some of these acids and the higher relative distribution of acetic acid among the allergic infants may be an indication of an imbalance in, or a delayed development of the gut flora, which may disturb the development of a normal Th1-/Th2-balance. It might also be tempting to speculate that the low levels of butyric acid may influence the maturation of the colonocytes [27].

The levels of the other MACs analysed in this study, conversion of cholesterol to coprostanol and tryptic activity, also agreed with those found in earlier studies [28]. None of these factors, however, differed significantly between the two groups.

Although, no associations between breast feeding or antibiotic treatments and the composition of SCFAs were found in this study, these factors do influence the gastrointestinal flora and thereby also the composition of SCFAs in faeces. However, for breast feeding, the effect is strongest during the first 9 months of life. At 12 months of age, when the children are not exclusively breast-fed anymore, breast feeding do no longer influence the SCFA

composition to a significant extent [29]. The median age of the children in our study was 13 months (range 11–18) when the faeces samples were collected and they were exclusively breast-fed for a median time period of 4 months (range 0–6). Antibiotic treatment may also influence the composition of SCFAs in faeces at least if the antibiotic treatment is taken within 3 months before collection of the faeces sample [29]. None of the children in our study received any antibiotic treatment within this time period before the faeces sample was collected. Furthermore, as the diet and the use of antibiotic were similar in the allergic and nonallergic infants, these factors cannot explain the observed differences in the composition of the microbial flora.

In conclusion, the results indicate an imbalance in the gut flora of allergic infants. As the microbial flora is driving the maturation of the immune system, changes in its composition, as a consequence of an altered lifestyle and diet in industrialized societies, may play a role for the higher prevalence of allergy.

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