INTESTINAL MICROFLORA

Aberrant composition of gut microbiota of allergic infants: a target of bifidobacterial therapy at weaning?

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Background: Recent data have outlined a relationship between the composition of the intestinal microflora and allergic inflammation, and demonstrated the competence of probiotics in downregulation of such inflammation.

Aims: Our aims were to characterise the relationship between gut microbes and the extent of allergic sensitisation and to assess whether the efficacy of bifidobacterial supplementation in the treatment of allergy could relate to modulation of the intestinal microbiota.

Methods: This randomised study included 21 infants with early onset atopic eczema of whom eight were intolerant (highly sensitised group (HSG)) and 13 tolerant (sensitised group (SG)) to extensively hydrolysed whey formula (EHF). In the SG, six were weaned to EHF without (placebo group (PG)) and seven to EHF with *Bifidobacterium lactis* Bb-12 supplementation (bifidobacteria treated group (BbG)). The faecal microflora of infants in the HSG was analysed only before weaning whereas in the SG the faecal microflora was analysed both before and after weaning.

Results: Infants in the HSG had greater numbers of lactobacilli/enterococci than those in the SG. Serum total IgE concentration correlated directly with *Escherichia coli* counts in all infants and with bacteroides counts in the HSG, indicating that the presence of these bacteria is associated with the extent of atopic sensitisation. The effect of supplementation was characterised as a decrease in the numbers of *Escherichia coli* and protection against an increase in bacteroides numbers during weaning.

Conclusions: These data indicate that bifidobacterial supplementation appears to modify the gut microbiota in a manner that may alleviate allergic inflammation. Further studies are needed to confirm this conclusion.

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P robiotic therapy appears to alleviate allergic inflammation demonstrated as control of clinical symptoms and reduction of local and systemic inflammatory markers.[1][2] On the basis of experimental data, the mechanisms behind these outcomes may involve enhanced antigen elimination[3–7] and downregulated inflammatory responses via direct effects on the immune system[1][2][4][8–12] as well as effects seen as modified degradation, permeation, and presentation of food antigens.[13–16]

The mechanisms of the probiotic action may be mediated via effects on the microbial composition and metabolic activity of the intestinal flora.[17] Infants with food allergies have been reported to have a disturbed balance between beneficial and potentially harmful bacteria in the large intestine.[18–21] Such flora may provide a means for the initiation or perpetuation of allergic inflammation. Indeed, there are data to suggest that an aberrant microbial composition in the gut such as inadequate bifidobacterial biota may deprive the developing immune system from counterregulatory signals against T helper 2 mediated allergic responses.[19][21][23] Conversely, by triggering inflammatory responses or by toxin formation, some bacteria may increase gut permeability and thus exposure to potential allergens.[17][24–27] Of the predominant bacterial groups in infancy, some strains of *Escherichia coli*, bacteroides, and clostridia have such properties[28–30] and were therefore counted in the present study.

Substantial compositional changes in the intestinal microflora occur during weaning.[31] In this study our primary aim was to test whether the proven efficacy of a probiotic therapy to alleviate allergic inflammation during this period[2] could be associated with modifications to the microbiota. Also, the relationship between allergic sensitisation and composition of the microbiota was further evaluated.

METHODS

Subjects and study design

The study population comprised 21 infants from a cohort of 100 infants with early onset atopic eczema and/or gastrointestinal symptoms, family history of atopic disorders, and failure to thrive, as previously described.[32] We selected infants with a heightened risk of chronic allergic disease. They fulfilled the Hanifin criteria for atopic eczema during exclusive breast feeding[33] and had never been given any infant formula. At weaning, they (n=35) were randomised in a double blind manner to receive extensively hydrolysed whey formula (EHF; Valio Ltd., Helsinki, Finland) with (bifidobacteria treated group (BbG)) or without (placebo group (PG)) probiotic supplementation.

The clinical symptoms of 5/15 infants in the PG and 10/20 infants in the BbG were not improved; sustained symptoms included pruritus, eczema, gastrointestinal symptoms (such as loose stools, gas, and vomiting) and subjective symptoms reported by parents (for example, crying and restlessness). For ethical reasons weaning of these infants was continued with an amino acid derived formula (SHS International Ltd, Liverpool, UK) instead of EHF, as previous studies have identified that infants manifesting atopic eczema during breast feeding may be intolerant to EHF, possibly in response to the presence of residual milk proteins and/or aggregated or cross linked low

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molecular weight peptides.[34–38] These infants who were apparently intolerant to EHF are hereafter referred to as the highly sensitised group (HSG) and the infants who tolerated EHF as the sensitised group (SG).

Faecal samples were collected from infants in both the HSG and SG before weaning (that is, during exclusive breast feeding) at a mean (interquartile range (IQR)) age of 5.2 (4.2–6.0) months, and after weaning at a mean age of 9.l (7.8–10.5) months only in the SG. All infants whose faecal samples did not contain enough biomass for quantitative microbiological assessments were excluded from the study; the number of infants included in each group is presented in table 1.

The extent of sensitisation was evaluated by serum total IgE (Phadebas IgE Prist; Pharmacia, Uppsala, Sweden) and the severity of atopic eczema by the SCORAD method when faecal samples were collected.[39] At enrolment, median (IQR) serum total IgE concentration in the HSG was 11 (0–18) kU/l and SCORAD score 22 (12–25), and in the SG, 12 (6–23) kU/l and 9 (5–16), respectively. During weaning, cow's milk allergy was confirmed in 6/8 infants in the HSG and in 6/13 infants in the SG by a double blind placebo controlled challenge, as previously described.[32] The SCORAD score decreased in 3/6 infants in the PG and in 7/7 infants in the BbG (Fisher's exact p=0.07), supporting the previously published clinical report with a larger study population.[2]

The study protocol was approved by the Tampere University Hospital Committee on Ethical Practice and written informed consent to participate was obtained from the children's parents.

Probiotic supplementation

EHF was supplemented with *Bifidobacterium lactis* Bb-12 (Christian Hansen A/S, Hørsholm, Denmark) in a concentration of 1×10^9 colony forming units/g. A bifidobacterium strain was chosen for probiotic supplementation as bifidobacteria are considered important for infant health and suggested to be associated with a reduced risk of developing atopy.[19 40] *Bifidobacterium lactis* Bb-12 was originally isolated from the faeces of a healthy adult. This strain was selected due to its documented safety and efficacy in the prevention of rotavirus diarrhoea and strong in vitro adhesion properties indicative of the ability of temporary colonisation.[41–43] The dose was chosen based on studies on the required dose of probiotics in the prevention of diarrhoea.[41 44–46]

An independent microbiologist controlled the bacterial concentration using a standard plate count method. The formula was stored under dry and cool conditions. No significant decreases were observed in the numbers of bacteria in the test formulas during storage. Formula intake was assessed as previously described[2] and the mean daily intake of Bb-12 during the study period was defined as 8×10^{10} (range $6–11 \times 10^{10}$) colony forming units/kg body weight.

Analysis of the bacteriology of faecal samples using genetic probes

Parents scraped faecal specimens from the diapers after defecation. The specimens were immediately cooled at 6–8°C, delivered within 24 hours, and frozen at −75°C directly on receipt. Bacterial cells were harvested and fixed, and fluorescence in situ hybridisation (FISH) performed with fluorophore (indocarbocyanin Cy3) labelled oligonucleotide probes, as previously described.[19 47] Bifidobacteria were counted with probe BIF164 ('5-CATCCGGCATTACCACCC),[47] bacteroides with BAC303 ('5-CCAATGTGGGGGACCTT),[48] lactobacilli/enterococci with LAB158 ('5-GGTATTAGCA(T/C)CTGTTTCCA),[49] clostridia belonging to the *Clostridium histolyticum* group with HIS150 ('5-TTATGCGGTATTAATCT(C/T)CCTTT),[50] and *Escherichia coli* with EC1531 ('5-CACCGTAGTGCCTCGTCATCA).[51] The probe HIS150 detects a representative proportion of the known clostridia species
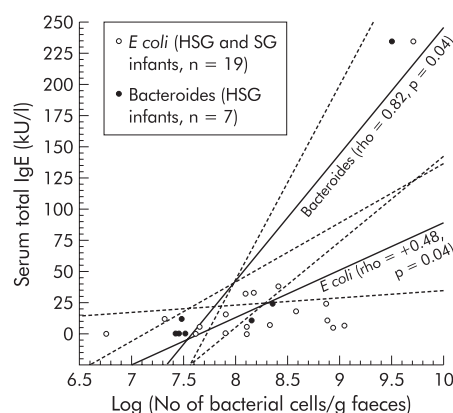

Figure 1 Scattergram showing faecal concentrations of bacteroides and *Escherichia coli* versus serum total IgE concentration in each individual. Regression lines with 95% confidence intervals (broken lines); Spearman's rho and p values are given for both slopes. HSG, highly sensitised group; SG, sensitised group.

commonly present in the infant gut—for example, *Clostridium paraputrificum*, *Clostridium butyricum*, and *Clostridium perfringens* but not *Clostridium difficile*.[52] All probes used in this study are among the best validated and most widely used oligonucleotide probes for FISH based analysis of faecal bacterial flora.[50 53] Total cell numbers were counted using a nucleic acid stain 4',6-diamidino-2-phenylindole. Cells were counted visually using a Leica Laborlux D epifluorescence microscope.

Statistics

We estimated that a total of 12 subjects were required for parallel comparisons of faecal bacterial numbers in the two groups to detect a difference at a two sided 5% significance level with over 80% probability if the true difference between the group means was at least 1 log.[54] This was based on the assumption that the standard deviation of the mean in bacterial numbers is 0.5 log, as indicated by preliminary observations.

Bacterial numbers are presented as median number of bacterial cells per gram of faeces (with IQR). Changes in bacterial numbers in faeces, which followed the intervention with infant formula, are presented as medians of relative changes: ((number of bacteria after weaning–number of bacteria before weaning)/the number of bacteria before weaning)×100%). The Mann-Whitney test was used to analyse intergroup differences in the bacterial flora between the SG and HSG before weaning as well as the relative changes between the PG and BbG during weaning. These changes were also analysed by Fisher's exact test to compare whether bacterial numbers tented to increase/decrease more frequently within the PG or BbG. Intragroup responses to weaning were analysed by the Wilcoxon signed rank test. The Spearman rank correlation (expressed as rho, describing linear relationships between two variables) was calculated to study the relationships between cell counts of different bacterial groups, serum total IgE, and SCORAD scores. Differences with p values <0.05 are reported as statistically significant. All analyses, except for the sample size calculation, were performed using computer software StatView for Windows version 4.5.7 (SAS Institute Inc., Cary, North Carolina, USA)

RESULTS

Gut microflora and the extent of allergic sensitisation

To evaluate whether the gut microbiota was associated with allergic sensitisation, the faecal flora before weaning was compared between the HSG and SG (table 1). Within the HSG, the lactobacilli/enterococci counts were significantly

Table 1 Bacterial numbers between infants in the highly sensitised group (HSG) and sensitised group (SG) before weaning, as well as changes in bacterial numbers in the SG during weaning, comparing the bifidobacteria treated group (BbG) and the placebo group (PG)

		No of cells/g faeces†		No of cells/g faeces: before/after weaning over change (%)‡	
		HSG before weaning (n=8)	SG before and after weaning (n=13)	BbG (n=7)	PG (n=6)
Bacterial group					
Bifidobacteria	×10⁹	31.4 (0.7–70.2)	9.2 (1.9–51.6)	17.1 (4.1–58.3)/5.3 (2.8–6.2) −82 (−92–24)%	5.6 (1.3–11.6)/2.4 (0.2–5.4) −68 (−92–13)%
Bacteroides	×10⁷	3.1 (2.8–16.4)	1.7 (1.1–5.8)	3.0 (1.5–152.9)/3.9 (2.8–6.3) **−31 (−65–122)%***	1.4 (1.1–1.8)/5.3 (1.8–41.4)§ **238 (100–690)%**
Lactobacilli/enterococci	×10⁷	**99.6 (34.4–290.0)***	**3.0 (0.8–17.0)**	1.1 (0.6–2.2)/0.6 (0.3–3.4) −72 (−91–317)%	23.5 (16.3–39.8)/32.4 (5.2–56.5) −36 (−77–64)%
Clostridium histolyticum	×10⁷	3.2 (0.9–17.4)	8.2 (5.8–14.0)	8.2 (4.5–25.6)/9.5 (7.2–35.6) 89 (−61–217)%	9.8 (6.2–13.5)/26.0 (13.3–29.0) 103 (−22–376)%
Escherichia coli	×10⁸	2.1 (0.4–7.5)	2.1 (1.3–5.4)	1.3 (1.3–3.0)/0.2 (0.1–1.0)§ **−87 (−93–60)%***	4.0 (1.8–9.8)/8.1 (2.4–16.0) **167 (−12–316)%**
Total cell counts	×10¹⁰	4.6 (2.4–8.3)	5.8 (2.0–9.9)	9.9 (4.1–17.0)/3.1 (1.7–9.0) −36 (−66–31)%	2.9 (2.0–5.3)/2.2 (1.3–3.2) −25 (53–89)%

†All values are expressed as group medians (interquartile range).
‡The change % expresses the change during weaning relative to the number of bacteria before weaning.
*Significant difference from the respective values compared (in bold) at p<0.05.
§Bacterial numbers after weaning were significantly (p=0.04 for bacteroides and 0.02 for *E coli*) different from those during breast feeding.

(p=0.002) higher than in the SG. In the HSG, the faecal concentration of bacteroides correlated directly with the total concentration of IgE in serum (fig 1) but not in the SG (rho= −0.28, p=0.35). In the whole study population (n=19), the total concentration of IgE in serum correlated directly with the numbers of *E coli* in faeces (fig 1).

Effects of bifidobacterial supplementation on the gut microbiota

The response of the digestive microflora to bifidobacterial supplementation was studied by comparing changes in the faecal microbial composition during weaning between the PG and BbG. As illustrated in table 1, the changes in the bacteroides and *E coli* flora were significantly (p<0.05) different between these infant groups. Within the PG, the faecal bacteroides numbers increased in all, and *E coli* numbers in 4/6 infants, while in the BbG bacteroides numbers decreased in 4/7 and *E coli* numbers in all infants (p=0.07 and 0.02, respectively, for bacteroides and *E coli*).

DISCUSSION

Bifidobacterial supplementation prevented the increase in the numbers of bacteroides and *E coli* during weaning, which is in agreement with previous in vitro and clinical data.[55–58] We postulate that this effect may be part of the mechanism by which bifidobacterial supplementation alleviates atopic eczema.[2] A similar effect may also contribute to the protective effect exclusive breast feeding has been suggested to have in high risk infants[59]; the gut flora of breast fed neonates is essentially dominated by bifidobacteria while formula fed infants exhibit a more complex flora with relatively high numbers of bacteroides and *E coli*.[53]

In support of our postulation, in the present study high numbers of bacteroides and *E coli* were associated with the extent of atopic sensitisation. Whether *E coli* and bacteroides may actually promote atopic sensitisation remains to be verified. Our recent data tentatively attest to this possibility. In one study comprising 15 infants with early onset atopic eczema, those six who were later diagnosed as allergic to cow's milk tended to have the highest bacteroides counts during exclusive breast feeding.[21] Our unpublished data on 69 infants with cow's milk allergy showed that of those infants who had a detectable concentration of IgE in serum, 74% (36/51) had *E coli* as part of their most predominant culturable aerobic flora

during breast feeding while among infants in whom serum IgE was not detected the respective value was only 39% (7/18) (Kirjavainen *et al*, unpublished). Conversely, others have shown that oral introduction of a non-enteropathogenic *E coli* strain after birth can reduce the risk of developing allergic disease.[60] This discrepancy is in agreement with previous evidence suggesting that the endotoxin lipopolysaccharide (LPS) reduces the risk of development of atopic disease by stimulating Th1 cells while in asthmatic patients LPS aggravates bronchial inflammation and thus the symptoms of the disease.[27] Similarly, in infants with atopic eczema, *E coli* (for example, due to LPS) may evoke an inflammatory response in the gut leading to increased allergen uptake and thereby greater atopic sensitisation.

The higher lactobacilli/enterococci counts in the HSG than in the SG tentatively demonstrates an association between these bacterial genera and the extent of allergic sensitisation. This suggestion is in agreement with our previous data demonstrating a direct correlation between lactobacilli/enterococci counts and serum total IgE concentration in atopic infants who had been weaned to amino acid derived infant formula. A number of other studies however have found that lactobacilli are associated with beneficial effects in the management of atopic eczema[1 2 61] and cow's milk allergy.[9 14–16] On this basis it seems unlikely that robust lactobacillar flora would have allergic sensitisation promoting properties. Some species of enterococci have virulence factors that can compromise the gut barrier and in theory could thereby affect atopic sensitisation but to our knowledge no previous data have been reported that would support this theory.[62] A conceivable explanation is that allergic sensitisation promotes the growth of lactobacilli and/or enterococci by causing changes in the gut ecology. Such an effect could be secondary to the disease; for example, the increase in the numbers of oral lactobacilli in asthmatic children has been suggested to be a response to their medication.[63]

In conclusion, bifidobacterial supplementation modulates the composition of the gut microbiota during weaning in a manner that could contribute to alleviation of the symptoms of atopy. The desirable modifications were identified as restraining of bacteroides and *E coli* flora, but unexpectedly also the desirability of a robust lactobacillar/enterococcal flora was called into question. Detailed analyses of the microbial composition of allergic infants at different ages and at the

species level are needed for a better understanding of the significance of the microbiota associated abnormalities in allergy. Such studies should also provide the means for more accurate targeting and thus more effective and better controlled modulation of the microbial milieu.

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