

An anthroposophic lifestyle and intestinal microflora in infancy

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The intestinal flora is considered to have an impact on the development of the immune system. In the anthroposophic lifestyle, a diet comprising vegetables spontaneously fermented by lactobacilli, and a restrictive use of antibiotics, anti-pyretics and vaccinations, is typical. The aim of this study was to assess the gut flora in infants in relation to certain lifestyle characteristics associated with anthroposophy. Sixty-nine children < 2 years of age with an anthroposophic lifestyle, and 59 infants of a similar age with a traditional lifestyle, were clinically examined and questionnaire replies assessed. Fecal samples were analyzed by bacterial enumeration, bacterial typing through biochemical fingerprinting and by measuring microflora-associated characteristics (MACs). The numbers of colony-forming units (CFU)/g of feces were significantly higher for enterococci and lactic acid bacteria in children who had never been exposed to antibiotics (5.5×10^7 vs. 2.1×10^7 ; $p < 0.001$ and 10×10^7 vs. 4.1×10^7 ; $p < 0.01$, respectively). Furthermore, the number of enterococci was significantly higher in breastfed and vegetarian infants ($p < 0.01$). The diversity (Simpson's diversity index) of lactobacilli, as determined by biochemical fingerprinting, was higher in infants born at home than in those born in hospital ($p < 0.01$). Several MACs were related to specific lifestyle features, and infants with an anthroposophic lifestyle had a higher proportion of acetic acid and a lower proportion of propionic acid in their stool as compared to the control children. In conclusion, lifestyle factors related to the anthroposophic way of life influenced the composition of the gut flora in the infants. These differences may contribute to the lower prevalence of atopic disease previously observed in children in anthroposophic families.

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The establishment of intestinal microflora is a major factor driving the maturation of the immune system in newborns (1). Studies on germ-free animals indicate that the establishment of gut flora is a prerequisite for development of oral tolerance and immune deviation (2). It is thus postulated that a lack of certain bacteria, colonization with inappropriate strains, or a limited bacterial turnover, could delay the development of tolerance in infancy (3). Several bacteria, mainly belonging to the genus *Lactobacillus*, are used as probiotics. This term is defined as 'a live microbial feed supplement that beneficially affects the host by improving the

intestinal microbial balance' (4). Dietary habits and environmental conditions influence the microbial pattern of the gut flora. Thus, supplied probiotic strains are detectable in stool (5). Furthermore, oral antibiotics cause changes in ecology (6) and predicted subsequent atopic disease in infancy (7) congruent with their ability to induce a T helper 2 (Th2)-polarized immune response (8). The possible role of intestinal microflora in the development of atopic allergy is further highlighted in epidemiologic studies where immunoglobulin E (IgE)-mediated allergy was found to correlate with differences in the composition of fecal short-chain fatty acids (9)

and higher counts of aerobic bacteria (10). Furthermore, lactobacilli used as probiotics were shown to prevent atopic disease (4) and to inhibit murine IgE production (11).

Recently, a lower prevalence of atopy was found among children with an anthroposophic lifestyle, as compared to other children living in the same area (12). An anthroposophic lifestyle comprises a high intake of organically produced food items, including spontaneously fermented vegetables containing live lactobacilli. In addition, a restrictive use of antibiotics, anti-pyretics and vaccinations is common in these families (12).

The aim of this study was to test whether the microflora of the gut in infants was affected by certain lifestyle characteristics associated with anthroposophy. As there is no general method for using to assess the intestinal microflora, several detection methods were used in parallel.

Subjects and methods

Study population and design

All families with children < 2 years of age who were enrolled in an anthroposophic Child Welfare Center (CWC) and living in a municipality located 60 km south of Stockholm, Sweden, were invited to participate during March–June 1999. Infants from a traditional CWC in the same municipality and who were born during the same time-period were randomly sampled from the CWC register. All parents received basic information about the investigation by post. Those who agreed to participate were sent a questionnaire regarding social and environmental variables, including food habits, history of infectious diseases, and anti-microbial treatments, as well as atopic symptoms of the child and its family. The information on vaccinations was verified by comparison with CWC documents, which were available for all infants.

Definitions

Two doctors (J.S.A., J.S.) jointly met all the infants at their CWCs for clinical examination and assessment of questionnaire replies. Each child was classified as clinically atopic or healthy with respect to asthma, atopic dermatitis, and food allergy. Bronchial asthma was defined as three or more episodes of wheezing when older than 3 months of age, or any episode of wheezing if combined with asthma in the family or other atopic symptoms in the child, or no recovery when infection ceased. Atopic dermatitis was

defined and scored according to the criteria used in a previous study (12). Food allergy was defined as acute onset of symptoms such as skin reactions, wheezing, vomiting or diarrhea on more than one occasion after ingestion of or contact with a particular food. A summary measure included 15 characteristics of the anthroposophic lifestyle ('Steiner-units') (12), defined as no MMR vaccination (against measles, mumps, and rubella), none of seven other vaccinations before 6 months of age, antibiotics not more than twice and not before 2 years of age, anti-pyretics not more than twice and not before 6 months of age, regular consumption of fermented vegetables, consumption of fermented vegetables for at least 1 year, and consumption of mainly organic or biodynamically produced food (organically by anthroposophical methods) in early childhood.

Fecal and food samples

A fecal sample was obtained from each infant within 30 min of defecation, according to instructions given orally and in writing to the parents. The specimen, usually collected from the nappy, was distributed in two plastic tubes into 5- and 20-ml samples, frozen at home within 10 min at -20°C , and delivered in a frozen state to the laboratory within 14 days for storage of up to 5 months at -70°C and -20°C , respectively. The analyses were performed blind with regard to CWC source. Non-pasteurized fermented food items, such as spontaneously fermented vegetables and dairy products, were sampled ($n = 6$) from local shops and stored at $+4^{\circ}\text{C}$ for a maximum of 6 months.

Bacteriological analyses

Culture. The 5-ml fecal samples were thawed and diluted 10^{-1} in a freezing medium containing phosphate buffer, pH 7.1, supplemented with albumin (25 g/l), sucrose (74 g/l), and sodium glutamate (0.6 g/l) (KS Substrate; Karolinska Hospital, Stockholm, Sweden). Further 10-fold dilutions of the fecal samples were performed in phosphate-buffered saline (PBS) (pH 7.4) to 10^{-5} , and 0.1 ml of each dilution was spread onto appropriate agar plates. The number of coliform bacteria was estimated by counting colonies on cystine lactose electrolyte deficient (CLED) agar (LabM, Lancashire, UK) and the number of enterococci estimated by counting colonies on Enterococcus agar (Difco Laboratories, Detroit, MI, USA) after aerobic incubation at 37°C for 24 h. The number of lactic acid-

producing bacteria was counted on Rogosa agar (Merck, Darmstadt, Germany) after anaerobic incubation at 37°C for 96 h. Potential *Clostridium difficile* colonies were picked from taurine cycloserine cefoxitin fructose agar (TCCFA) plates (13) after anaerobic incubation at 37°C for 48–72 h, re-cultured on blood agar (anaerobic incubation) and identified as *C. difficile* by typical colony and microscopic appearance. From each fecal sample, coliform bacterial colonies ($n = 16$) and *Enterococcus*-like colonies ($n = 8$) were randomly picked from respective agar plates and re-cultured, prior to typing by biochemical fingerprinting. For biochemical fingerprinting of *Lactobacillus* spp., the samples were cultured on de Man, Rogosa and Sharp (MRS) agar (*Lactobacillus* agar; Merck) supplemented with vancomycin (20 g/l) (Sigma Co., St Louis, MO, USA) and incubated anaerobically at 37°C for 72 h. Eight lactobacilli colonies from each sample were then re-cultured on MRS plates for further analysis. Six food samples (locally produced fermented cabbage ($n = 2$); fermented carrots; sour milk and yoghurt; and commercially produced unpasteurized fermented cabbage) were homogenized and diluted 10^{-1} in PBS using a stomacher. The suspensions were further diluted in PBS to 10^{-6} and 0.1 ml was spread on Rogosa agar plates supplemented with vancomycin (20 g/l). After anaerobic incubation at 37°C for 72 h, eight colonies of *Lactobacilli* were picked from each sample and re-cultured for biochemical fingerprinting analysis, as described above for the fecal samples.

Biochemical fingerprinting of bacterial isolates

The diversity of the three bacterial groups – coliforms, enterococci and lactobacilli – were investigated by picking 8–16 typical isolates per sample of each group and typing them to below the species level using the PhenePlate™ (PhP) biochemical fingerprinting method (PhPlate Microplate Techniques AB, Stockholm, Sweden) (14). The PhP-RE plates were used for coliform isolates, and the PhP-RF (15) plates were used for enterococcal isolates. For lactobacilli, a new set of PhP plates (PhP-LB plates) were developed in the laboratory of one of the authors (R.M.). The diversities within the bacterial groups in each sample were calculated as Simpson's diversity index (16) and the similarities between the investigated populations were calculated as the population similarity index (S_p) (17). In addition, the occurrence of isolates of *Lactobacillus* spp. belonging to the same PhP type, and that were found both in the infant's stool and in the food samples, was assessed.

Metabolic activity of fecal samples. The same fecal suspension as cultured was distributed into the wells (150 μ l) of a PhP-48 microplate, which contains 48 different reagents (18). The plate was incubated at 37°C and fermentative activities read at different time-points up to 48 h of incubation. A metabolic activity index (18) was obtained through calculation of a mean fermenting score (0–1) for all the 48 fermentations. In addition, a pattern of fermentative activity in 48 tests for each sample was produced, which was used to calculate similarities between pairs of samples. Thus, a mean similarity coefficient, based upon the similarity coefficients obtained with all possible pairwise combinations, was calculated for each of the two lifestyle groups.

Microflora-associated characteristics. The 20-ml fecal samples were thawed and homogenized for determination of six microflora-associated characteristics (MACs) (19), listed below.

The short chain fatty acid pattern. In order to evaluate the interplay between the host and the microflora, the short chain fatty acid (SCFA) pattern was determined. Aliquots of 0.5 g of feces were homogenized in distilled water containing 2-ethylbutyric acid and H_2SO_4 as an internal standard (Sigma-Aldrich, Stockholm, Sweden). The homogenate was vacuum distilled and analyzed on a gas chromatograph (Perkin-Elmer auto system XL; Perkin-Elmer, Palo Alto, CA, USA) (20). The concentrations of SCFAs were calculated on a Turbochrome auto-analyzer system (Perkin-Elmer) and expressed as relative distribution of each SCFA. Each series started and ended with a standard solution. The detection level was 0.2 mmol/kg of feces.

Inactivation of tryptic activity (FTA), degradation of β -aspartylglycine and mucin. Aliquots of 0.5 g of feces were mixed with twice the amount of saline, incubated at 4°C for 2 h, centrifuged at 4500 g, and the supernatant used. Inactivation of FTA, reflecting complex interactions between pancreas-derived trypsinogen and intestinal micro-organisms, was determined using spectrophotometry (HITACHI 150-20 Spectrophotometer; Hitachi, Tokyo, Japan) in parallel with blanks at 405 nm after using *N*-benzoyl-DL-arginine-4-nitroanilide hydrochloride (BAPNA) (Sigma-Aldrich) as reagent (21). The detection level was 3 μ g/g of feces. Degradation of β -aspartylglycine, reflecting the so-called colonization resistance, was analyzed by high-voltage paper electrophoresis using pure β -aspartylglycine (3 μ g/ml) (Bachem, Bubendorf, Switzerland) as a positive control (22). Degradation of mucin, in order to study the integrity of the intestinal

mucosa, was analyzed using agar-gel electrophoresis (19).

Conversion of cholesterol to coprostanol. The conversion of cholesterol to coprostanol was studied to investigate hepatic and intestinal microbial interplay. The residual supernatant from the above procedure and the sediment were mixed with twice the volume of NaOH (10 M)/ethanol, hydrolyzed at 60°C for 45 min, and extracted with hexane for gas chromatograph analysis (23) (Hewlett Packard, Palo Alto, CA, USA). The results were expressed as percentage coprostanol relative to the total amount of cholesterol and coprostanol. Peak areas of < 5% were considered as impurities and disregarded.

Conversion of bilirubin to urobilinogens. Hepatic and intestinal microbial interplay was investigated by studying the conversion of bilirubin to urobilinogens. Aliquots of 0.7 g of feces were analyzed using a spectrophotometric method, applying the Ehrlich aldehyde reaction with *p*-dimethylaminobenzaldehyde directly to an aqueous extract of feces, followed by reduction of urobilin to urobilinogens by ferrous hydroxide (24).

Ethical aspects

The study was approved by the local ethics committee in south Stockholm. All parents were supplied with information orally and in writing, and the parents gave informed consent before entering their children into the study.

Statistics

To assess the diversities, similarities, and metabolic activities following biochemical fingerprinting, calculations were performed using the PhP Software (PhPlate Microplate Techniques AB). The data were further analysed using Stata 7.0 (Stata Corporation, College Station, TX, USA). Comparisons between lifestyle characteristics and results from fecal analyses were made using the Mann-Whitney *U*-test. For dichotomous data, the chi-square test, Fisher's exact test or logistic regression were used. Regression analyses were performed for outcome variables, showing normal distribution. Only *p*-values of < 0.01 were considered statistically significant; however, higher *p*-values are also given. Odds ratios (OR), coefficients (Coef) and 99% confidence intervals (CI) were estimated from the regression analyses. To assess age-dependent differences in fecal composition, the children were divided into two age-groups (< 12 and > 12 months of age).

Results

Characterization of the study groups

At the anthroposophic CWC, all 61 infants were invited and two families (3%) refused to participate, as compared to 11 families among the 82 controls (13%). Two infants in the anthroposophic group were excluded because of chronic diseases that had required continuous antimicrobial treatment since the neonatal period. Therefore, the total study group comprised 128 infants between 1 and 24 months of age. None of the infants at the anthroposophic CWC had fewer than 10 exposure characteristics of an anthroposophic lifestyle (12). At the traditional CWC, 12 children had more than 10 of these characteristics, indicating an apparent anthroposophic lifestyle. They were therefore included in the anthroposophic group in the subsequent analyses. Accordingly, the final groups consisted of 69 infants with an anthroposophic lifestyle and 59 with a conventional lifestyle (Table 1).

The average number of children in the families (1.9) was similar in the two groups, as well as birth order (1.9). Delivery at home occurred exclusively in the anthroposophic families and if the child was delivered in hospital, it was less frequently by Caesarean section in this group (Table 1). The mothers in the anthroposophic group tended to breastfeed for a longer time-period, and they consumed biodynamic and/or organic food more often during pregnancy and the lactation period compared to the control mothers (data not shown). A highly vegetarian diet, based on biodynamic and/or organic food, was more common in the anthroposophic infants as compared to the controls. Unpasteurized cow's milk was rarely consumed in either group (*n* = 10). However, spontaneously fermented vegetables were common in the diet of the anthroposophic families. Antibiotics, mainly β -lactam antibiotics, and anti-pyretics (primarily paracetamol) were less frequently used by infants with an anthroposophic lifestyle, and they had later and fewer vaccinations than the controls. In addition, furred pets tended to be less common in the households of anthroposophic families, and parental ethnicity tended more often to be non-Caucasian than in the control group. The prevalence of atopic heredity by history and the number of smoking mothers were similar in the two groups. In the anthroposophic group one infant had current antibiotic treatment compared to four control infants. No infant showed signs of gastroenteritis at the time of fecal sampling.

In the anthroposophic group, six infants (9%) were diagnosed as atopic compared to nine

Table 1. Demographic data and exposures of infants with an anthroposophic lifestyle and controls

Characteristics	Anthroposophic lifestyle (n = 69)	Controls (n = 59)
Age (months)*		
1–12	31 (44.9)	21 (35.6)
12–24	38 (55.1)	38 (64.4)
Female	29 (42.0)	25 (42.4)
Born at home	15 (21.7)	0
Birth by Caesarean section	3 (4.3)	11 (18.6)
Breastfed, still exclusively	15 (21.7)	5 (8.5)
Breastfed, still to any extent	30 (43.5)	19 (32.2)
Vegetarian food	41 (59.4)	5 (8.5)
Food mainly organic/biodynamic†	61 (88.4)	11 (18.6)
Unpasteurized cow's milk	8 (11.6)	2 (3.4)
Fermented vegetables‡		
Child, more than once/month	15 (21.7)	1 (1.7)
Family, more than once/month	33 (47.8)	2 (3.4)
Antibiotics, never†	60 (87.0)	33 (55.9)
Anti-pyretics, never†	61 (88.4)	8 (13.6)
No vaccination before 6 months of age‡	67 (97.1)	8 (13.6)
No vaccination at all†‡	55 (79.7)	3 (5.1)
Furry pet at home, ever	25 (36.2)	33 (55.9)
Non-Caucasian parent	11 (15.9)	3 (5.1)
Parental atopy, by history		
Mother	10 (14.5)	7 (11.9)
Father	11 (15.9)	13 (22.0)
Both	4 (5.8)	5 (8.5)
Maternal smoking (≥ 1 cigarette/day)	9 (13.0)	10 (16.9)

Data are expressed as n (%).

*At fecal sampling.

†Exposures common in children of anthroposophic families (see the text).

‡Measles, mumps, and rubella vaccination (recommended from 18 months of age) was given to none of 16 children in the anthroposophic group ≥ 18 months of age, compared to six of 22 among the controls ≥ 18 months of age.

(15%) in the control group. In both groups five children had atopic dermatitis, the severity of which scored similarly. In the anthroposophic group, one child had asthma compared to five children among the controls. One infant in the control group had a history of allergy to peanuts.

Bacteriological analyses

Culture. Fecal samples were obtained from all 128 children. Coliform bacteria were isolated in 83%, enterococci in 97%, and lactic acid bacteria in 96% of the samples (Table 2). In infants younger than 12 months of age, coliforms and enterococci were isolated more often in the fecal samples from the control infants, whereas lactic acid bacteria were present more often in samples from the anthroposophic group. However, none of the differences were statistically significant. Among the positive samples, the numbers of colony-forming units (CFU)/g of feces were

similar in the anthroposophic group of infants and controls (Table 2). However, children older than 12 months of age had lower counts of enterococci as compared to younger infants, and this was most obvious in the control group (Table 2). On the other hand, a higher number of enterococci were found in breastfed and vegetarian infants (Fig. 1). The number of lactic acid-producing bacteria was not affected by a diet that included fermented vegetables, either by the infant (Fig. 1) or by the family (data not shown). Infants who had at some time been treated with antibiotics (even if the treatment had been given more than 12 weeks before fecal sampling) had fewer enterococci than infants who had never been given antibiotics ($p < 0.001$). This was also seen among older children who had at some time been treated with anti-pyretic drugs, as compared to children of the same age-group who had never received anti-pyretics. The number of lactic acid bacteria was significantly lower among infants who had at some time been treated with antibiotics, as compared to those who had never been exposed. *C. difficile* was rarely detected ($n = 8$), especially in the anthroposophic group (data not shown).

Diversities and similarities. Typing data for 3,453 isolates (1,675 coliforms, 843 enterococci and 935 lactobacilli) were obtained by biochemical fingerprinting. The diversity in each sample and the population similarities between samples taken from the same age-group were calculated. The diversity of coliforms and enterococci was similar among the anthroposophic infants and the controls (Table 2). Younger infants (< 12 months of age) tended to have greater diversity in their coliform population than older children. This was most pronounced among the controls ($p = 0.02$). The enterococci were slightly less diverse in the younger children; however, the differences were again most pronounced in the control group ($p = 0.07$). In contrast, the lactobacilli tended to be more diverse among the anthroposophic infants, as compared to the control infants ($p = 0.03$), and this diversity remained high also after 12 months of age, in contrast to the controls (Table 2). Breastfeeding was not associated with any particular degree of bacterial diversity (data not shown). Moreover, the diversity among lactobacilli was greater in infants born at home ($p < 0.01$) than in those born in hospital, even after excluding babies born by Caesarean section ($p < 0.01$). This difference in diversity seemed to persist beyond 12 months of age ($p = 0.03$, $n = 7$) (data not shown). The diversity of lactobacilli also tended to be higher

Table 2. Colony-forming units (Cfu) of certain bacteria, and diversity among bacterial strains in infants with an anthroposophic lifestyle and in controls

Group	Age (months)	Samples (n)	Coliform bacteria			Enterococci			Lactic acid bacteria/lactobacilli		
			Positive samples n (%)	Mean no. of log CFU/g of feces	Mean diversity index	Positive samples n (%)	Mean no. of log CFU/g of feces	Mean diversity index	Positive samples n (%)	Mean no. of log CFU/g of feces	Mean diversity index
Anthroposophic	< 12	31	23 (74)	5.79	0.49	29 (94)	6.91	0.50	30 (97)	7.00	0.61
	≥ 12	38	32 (84)	5.34	0.46	37 (97)	6.18	0.54	37 (97)	7.04	0.60
	All	69	55 (80)	5.53	0.47	66 (96)	6.49	0.52	67 (97)	7.02	0.60
Controls	< 12	21	19 (90)	5.41	0.61	21 (100)	7.11*	0.44	19 (90)	7.58	0.56
	≥ 12	38	32 (84)	5.23	0.42	37 (97)	5.30*	0.56	37 (97)	7.20	0.42
	All	59	51 (86)	5.30	0.49	58 (98)	5.95	0.52	56 (95)	7.32	0.50
All	< 12	52	42 (81)	5.61	0.54	50 (96)	7.00†	0.47	49 (94)	7.23	0.59
	≥ 12	76	64 (84)	5.28	0.43	74 (97)	5.74†	0.55	74 (97)	7.11	0.51
	All	128	106 (83)	5.41	0.48	124 (97)	6.26	0.52	123 (96)	7.18	0.54

*†p < 0.01 (Mann-Whitney U-test).

	Bacteria (Cfu/g faeces)			Microflora associated characteristics, MACs (relative distribution)									
	Coliforms	Enterococci	Lactic acid bacteria	Acetic acid	Propionic acid	i-Butyric acid	Butyric acid	i-Valeric acid	Valeric acid	i-Caproic acid	Caproic acid	Coprost-anol	Urobilinogen
total n=128													
Anthroposophic lifestyle n=69				*** ↑	*** ↓	** ↓		*** ↓	** ↓				** ↓
Homeborn n=15											*** ↑		
Breastfed, still exclusively n=20		** ↑		*** ↑	*** ↓		*** ↓	*** ↓	*** ↓			*** ↓	*** ↓
Vegetarian food n=46		** ↑		*** ↑			*** ↓	** ↓	*** ↓			** ↓	*** ↓
Food mainly organic / biodynamic				*** ↑	** ↓	** ↓		*** ↓	*** ↓				** ↓
Fermented vegetables >1 time/month													
Antibiotics, ever n=35		*** ↓	** ↓	** ↓				*** ↑	*** ↑				** ↑
Antibiotics, but not for the last 12 weeks		*** ↓						**a ↑	** ↑				** ↑
Antipyretics, ever n=59 ^(a) (n=21) ^(b) (n=38)		**b ↓		*** ↓	** ↑	** ↑	***a ↑	*** ↑	*** ↑			**a ↑	** ↑
Vaccination, any n=70				*** ↓	** ↑			** ↑	*** ↑				*** ↑

non significant

** p <0.01; *** p <0.001 (Mann-Whitney U-test).

↑ Elevated compared to non-exposed.

↓ Lowered compared to non-exposed.

^a Children <12 months of age.

^b Children >=12 months of age.

non significant

** p < 0.01; *** p < 0.001 (Mann-Whitney U-test).

↑ Elevated compared to non-exposed.

↓ Lowered compared to non-exposed.

^a Children <12 months of age.^b Children ≥12 months of age.

Fig 1. Correlation between fecal analyses and certain lifestyle exposures related to an anthroposophical environment.

in the group of infants with a diet that regularly comprised fermented vegetables ($p = 0.03$). Use of antibiotics by the infants did not, however, affect any of the bacterial diversities (data not shown). Different infants in the anthroposophic group seemed to carry identical strains of lactobacilli to a higher extent than infants in the control group (the mean S_p was 0.08 and 0.03, respectively). No such differences in population similarities were seen for the coliforms and enterococci (data not shown).

Cultures from the food samples confirmed the presence of *Lactobacillus plantarum*. A comparison was made between lactobacilli phenotypes

found in the fecal and in the food samples. Two infants in the anthroposophic group carried a *Lactobacillus* strain of the same phenotype as that of a food sample; in one infant the strain matched the phenotype of a certain fermented cabbage, in the other infant it matched the yoghurt used in the anthroposophic community (data not shown). The mean metabolic activity index, defined as the sum of fermentative activities against 48 different reagents (18), was estimated for each fecal sample. The older group of control infants tended to have a higher mean metabolic activity index than those younger than 12 months of age (0.32 vs. 0.22, $p = 0.02$). This

difference was not found in infants with an anthroposophic lifestyle (mean metabolic activity index 0.26 vs. 0.28; $p = 0.53$).

Microflora-associated characteristics. Samples from all 128 infants were analyzed for MACs. Statistical significance tests for some MACs, in relation to certain exposure characteristics, are shown in Fig. 1. The proportion of fecal SCFAs, except i-butyric, i-caproic and caproic acid, correlated well with breastfeeding. The proportion of acetic acid was higher in anthroposophic infants, even after adjustment for exclusive breastfeeding and age (Coef 8.0; 99% CI: 2.5–13.4). Furthermore, anthroposophic infants had a lower proportion of propionic acid, as compared to the controls, also after adjustment for exclusive breastfeeding, vegetarian food and age (Coef –5.1; 99% CI: –10.1 to –0.14). Acetic and propionic acids showed a similar correlation to the consumption of organic food, no use of anti-pyretics and to restrictive vaccination (Fig. 1). The proportion of i-valeric acid, valeric acid, and excretion of urobilinogen were associated with exposures such as consumption of vegetarian or organic food, any use of antibiotics, any use of anti-pyretics, and with vaccination. In non-parametric tests, i-caproic acid was not significantly correlated to any exposure under study (Fig. 1). However, logistic regression revealed that i-caproic acid was, by stepwise exclusion of potential confounders, independently more often present in those children never exposed to antibiotics (OR 3.3; 99% CI: 1.01–10.8), or ever given fermented food (OR 6.0; 99% CI: 1.44–25.3), and showed such a tendency in children who were still breastfed (OR 5.7; 99% CI: 0.99–32.7), as compared to children who were not. The presence of caproic acid strongly correlated to being born at home ($p < 0.001$) (Fig. 1), also by exclusion of those born by Caesarean section ($p < 0.001$), and this correlation tended to persist beyond 12 months of age ($p = 0.03$, $n = 9$) (data not shown). The metabolism of cholesterol was rarely detected in infants younger than 1 year of age. Thereafter, coprostanol was present in $\approx 66\%$ of the anthroposophic children and in $\approx 75\%$ of the control children, indicating a difference in the intestinal aerobic conditions. β -aspartylglycine appeared to be detected more often in fecal samples from the younger controls, and this is suggested to be related to their slightly earlier weaning. The tryptic activity levels were similar between the anthroposophic group and controls (data not shown). There was no relationship between atopy and any of the MACs, number or diversity of bacteria, or metabolic activity.

Discussion

Our data indicate that certain environmental exposures characteristic of an anthroposophic lifestyle, such as vegetarian food, a restrictive use of antibiotics and home birth, influence the composition of the intestinal microflora in infants. In agreement with previous studies, dietary factors (mainly breastfeeding and a vegetarian diet) were found to be associated with bacterial counts (25) and with indirect measures of bacterial metabolism, such as SCFAs, bilirubin, and cholesterol (19, 20, 23). The effect of age on the intestinal environment could only be adjusted for in some variables. However, this effect is assumed to be less important in the age-range (inter-quartile range 7.2–18 months) of children of the present study than during earlier infancy (20). The numbers of lactic acid-producing bacteria were similar in fecal samples from infants who regularly consumed fermented vegetables and those who did not. In contrast to previous studies, a diet containing fermented vegetables was not associated with higher levels of acetic and propionic acids in the stool (5), but with i-caproic acid. The presence of i-caproic acid has been reported to be associated with the presence of *C. difficile* (9), but not to consumption of fermented vegetables. Furthermore, *C. difficile* was only isolated from one infant whose diet included such food. Moreover, an increased concentration of propionic acid, which has been associated with high counts of lactobacilli, could not be confirmed among infants with such a diet. This could indicate a limited intake of lactobacilli-containing food items by the children. However, the similar bacterial diversity among younger and older anthroposophic infants might indicate a more stable and 'mature' composition of their intestinal flora at an earlier age in life. This is also suggested by the tendency towards a more persistent mean metabolic activity in the feces from infants in the anthroposophic group.

A persistent difference in the intestinal flora is reported in infants born by Caesarean section compared to infants delivered vaginally (26). In addition, we found that delivery at home, compared to vaginal delivery in the clinic, had an impact on intestinal microflora in infancy. The higher diversity in lactobacilli and the higher proportion of caproic acid seemed to persist beyond the first year of life. Moreover, a higher similarity of lactobacilli in children with an anthroposophic lifestyle was indicated. Taken together, this supports the assumption that characteristics such as home delivery and a high

intake of fermenting bacteria induce floras of *Lactobacilli* that show great diversity in each child and yet are more similar between the children within the group than the floras of children who live by the modern western lifestyle. This could indicate diversity being of importance as compared to administration of a single probiotic strain.

An impact of antibiotics on the number of enterococci and lactic acid bacteria in the fecal samples was found, and the duration of this effect was longer than described previously (6). Antibiotic treatment early in life thus appears to constitute a pressure on the microflora which could be substantial, and may also affect the maturation of the immune system (8). Certain antibiotics were thought to have greater impact on measures of the fecal samples than others, owing to differences in their antibiotic spectra (7). However, this could not be demonstrated statistically. Besides, the diversity of any bacterial group under study did not seem to be influenced by exposures such as antibiotic treatment or breastfeeding.

According to the 'hygiene hypothesis', the increasing prevalence of atopic diseases may be caused by major improvements in public health and personal hygiene, dietary changes, smaller families, and improved general living standards, i.e. a changed overall exposure to microbial stimulation (27). *Lactobacilli* are of certain interest in this regard (4, 11, 28). In the present study a tendency to greater diversity of *lactobacilli* was found in the anthroposophic group of infants. A high diversity is generally considered to represent a better biological function of a population, whereas a low diversity is normally associated with more restricted possibilities to adapt and a lowered resistance to pressure from the outside, implying a lowered colonization resistance. Low diversity could also be related to limited turnover, suggested to reduce the stimulation of the immune system (3). It can furthermore not be excluded that the use of antibiotics might constitute a contributory factor to the development of atopic allergy, if it causes such drastic microbial changes that they result in deviation of the immune response (8). It has been hypothesized that the lower prevalence of atopic disease among children of farmers correlates to high exposure to environmental microbes, e.g. from unpasteurized cow's milk (27). This food item was consumed exclusively by children classified as non-atopic in the present study. The relevance of the association between use of paracetamol, and several MACs and numbers of enterococci, is uncertain: sales of paracetamol

have been shown to correlate with atopic disease in schoolchildren (29) but those results are the subject of debate. Fever, however, may impede histamine-induced symptoms (30) and a correlation is found between early fever episodes in asthmatic children and lower reactivity in skin-prick testing (31). Thus, the influence of fever, anti-pyretics and antibiotics on immune regulation might be subjects for investigation in future studies. The finding of a strong correlation between caproic acid and infants born at home may encourage investigations in search of a microbial correlate.

Any assessment of the intestinal microflora from analyses of fecal samples has restrictions in validity, as the flora in the stool differs from that in the gut, especially the small intestine, owing both to internal milieu and to intestinal passage-time aspects (32). Besides, only those organisms capable of growing under laboratory conditions can be isolated. In this study, we therefore applied different methods for investigation of the bacterial flora. In addition to culture, the flora was characterized with regard to diversity of the three groups of bacteria studied using a biochemical fingerprinting system, and the metabolic activity of the fecal sample was determined (14). It should be noted that the metabolic activity test, as performed on a fecal suspension, probably measures both preformed and constitutive enzymes and, to a lesser degree, inducible and less active enzyme systems. In the biochemical fingerprinting, regulatory mechanisms of bacterial metabolism and bacterial transport systems for the substrate may also influence the outcome. Thus, the biochemical fingerprint and the metabolic activity may reflect the joint properties of a number of bacterial genes and their regulatory systems, explaining perhaps why biochemical fingerprinting yields such detailed highly discriminating results, often comparable to extensive genotyping methods (33). To assess differences between infants concerning intestinal microbes that have not yet been identified or that cannot yet be cultured, we applied functional studies of what biochemical reactions the intestinal microbes had already performed *in vivo* by applying the MAC concept. This approach has the advantages that it is not associated with problems in obtaining samples from areas of the gastrointestinal tract that are normally inaccessible (32). In conclusion, our study indicates that lifestyle factors related to the anthroposophic way of life correlate with differences in the gut flora of infants. This lifestyle is associated with a lower prevalence of atopy (12) and it involves several features, many

of which were more common in the general population some decades ago. These findings may provide clues to explain the recent increase in the prevalence of atopy in children subject to the modern western lifestyle. The development and composition of the intestinal microflora may be factors influencing the development of atopic manifestations.

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