RESEARCH ARTICLE

Impact of lifestyle on the gut microbiota of healthy infants and their mothers – the ALADDIN birth cohort

Helena M. Hesla[1,2], Fredrik Stenius[1,2], Lotta Jäderlund[3], Ronald Nelson[4], Lars Engstrand[5], Johan Alm[1,2] & Johan Dicksved[3,6]

[1]Sachs' Children and Youth Hospital, Södersjukhuset, Stockholm, Sweden; [2]Department of Clinical Science and Education Södersjukhuset, Karolinska Institutet, Stockholm, Sweden; [3]Department of Microbiology, Swedish University of Agricultural Sciences, Uppsala, Sweden; [4]Department of Clinical Sciences, Swedish University of Agricultural Sciences, Uppsala, Sweden; [5]Department of Microbiology, Tumour and Cell Biology, Karolinska Institutet, Stockholm, Sweden; and [6]Department of Animal Nutrition and Management, Swedish University of Agricultural Sciences, Uppsala, Sweden

Correspondence: Helena M. Hesla, Sachs' Children and Youth Hospital, Södersjukhuset, SE-118 83 Stockholm, Sweden.
Tel.: +46 8 6164000;
fax: +46 8 6164161;
e-mail: helena.marell-hesla@sodersjukhuset.se

Received 18 June 2014; revised 29 September 2014; accepted 30 September 2014. Final version published online 3 November 2014.

DOI: 10.1111/1574-6941.12434

Editor: Julian Marchesi

Keywords
454-pyrosequencing; anthroposophy; *Bifidobacterium*; breastfeeding; caesarean section; gut microbiota.

Abstract

An anthroposophic lifestyle, which has been associated with reduced allergy risk in children, has several characteristics that could influence gut microbiota. This study aimed to investigate the impact of anthroposophic lifestyle as well as specific early life exposures on the gut microbiota. In total, 665 stool samples from 128 mother–infant pairs from the ALADDIN birth cohort study were included. Samples collected from infants at ages 6 days, 3 weeks, 2 months and 6 months, and from their mothers before and after delivery, respectively, were analyzed using 454-pyrosequencing. Information regarding lifestyle exposures was collected prospectively through interviews and questionnaires. Six-month-old infants in anthroposophic families had a significantly higher abundance of *Bifidobacterium* and lower abundances of *Bacteroides* and *Veillonella*. Caesarean section and breastfeeding had a significant impact on the microbiota: caesarean section was primarily associated with delayed colonization of *Bifidobacterium* and *Bacteroides*, whereas breastfed children had a higher relative abundance of *Bifidobacterium* and a lower abundance of *Clostridiales*. However, despite large differences in lifestyle exposures, we determined no significant differences in the gut microbiota between the anthroposophic and non-anthroposophic mothers or their infants' before 6 months of age.

Introduction

Human gut microbiota is important for host health, especially as the early establishment of gut microbiota in a newborn infant is thought to be important for the development of the immune system (Penders *et al.*, 2007). Although an individual's genetic predisposition likely plays a role in this establishment (Zoetendal *et al.*, 2001; Dicksved *et al.*, 2008; Johansson *et al.*, 2011), it is highly influenced by environmental (lifestyle) factors (Dominguez-Bello *et al.*, 2011). The initial gut microbiota of infants delivered by caesarean section differs from that of vaginally delivered infants and resembles the skin microbiota rather than the mother's vaginal microbiota (Dominguez-Bello *et al.*, 2010, 2011). Breastfed infants have a gut microbiota that is more dominated by bifidobacteria and less diverse than formula-fed infants (Azad *et al.*, 2013a, b; Tannock *et al.*, 2013). The microbial diversity increases with age and in particular after the introduction of solid food (Penders *et al.*, 2006).

Researchers have for many years investigated the gut microbiota of small children in attempts to provide explanations for the great increase in immune-related diseases apparent during the last half-century. Despite the large number of studies on gut microbiota, differences in methodologies employed make comparisons and interpretations difficult. Both the culture-based and the earlier sequencing-based studies were time- and money-consuming and were therefore only performed on relatively small study cohorts. The development of high-throughput sequencing technologies has, together with new bioinformatics tools, enabled comparisons and analyses of the

FEMS MICROBIOLOGY ECOLOGY

composition and diversity of the microbiota with high coverage and in large sets of samples (Andersson *et al.*, 2008). This development has facilitated more comprehensive analyses of the microbiota in relation to health and diseases.

The composition of the microbiota has been linked to allergic disease and several studies have indicated a reduced abundance of lactobacilli and bifidobacteria as well as reduced diversity of gut microbiota in infants who later developed atopic eczema (Björksten *et al.*, 1999; Forno *et al.*, 2008; Wang *et al.*, 2008; Abrahamsson *et al.*, 2012). Interestingly, growing up in families with an anthroposophic lifestyle seems largely to protect children from developing allergic disease (Alm *et al.*, 1999; Flöistrup *et al.*, 2006; Stenius *et al.*, 2011). Among the characteristic factors of this lifestyle are: a high proportion of homebirths; fermented vegetables in the diet; restrictive use of antibiotics, antipyretics and vaccines; prolonged breastfeeding; a stress-reducing environment; and later daycare attendance (Stenius *et al.*, 2010, 2011). Many of these characteristic factors are of interest in allergy research and the ALADDIN (Assessment of Lifestyle and Allergic Disease During Infancy) birth cohort study was initiated to associate specific lifestyle factors with this allergy-protective effect. Earlier, cross-sectional studies indicated that the composition of the faecal microbiota of children who grow up in families with an anthroposophic lifestyle differs from that of controls (Alm *et al.*, 2002; Dicksved *et al.*, 2007).

In this study we used 454-pyrosequencing of 16S rRNA gene amplicons with the aim to investigate how the anthroposophic lifestyle affects the gut microbiota of healthy infants. We also related the gut microbiota of these infants to that of their mothers and to different lifestyle factors.

Materials and methods

Participants and sample collection

The families included in this study are part of the ALADDIN birth cohort. Individuals were recruited during pregnancy between September 2004 and November 2007 from anthroposophic and conventional Maternal Child Health Centres (MCHCs) in the Stockholm area. The study design and exposure characteristics have been thoroughly described previously (Stenius *et al.*, 2011). Demographic and lifestyle data were collected prospectively using questionnaires and interviews. Fecal samples were collected from the infants at ages 6 days, 3 weeks, 2 months and 6 months and from the mothers 1 week before delivery and when their child was 2 months, respectively. These samples were frozen within 20 min of collection and stored at −20 °C until later transport in a frozen state for storage at −70 °C.

Classification of the participating families into lifestyle groups was based on the choice of MCHCs and parental responses to three questions: (1) 'What kind of preschool/school will your newborn child probably go to?' (2) 'Have either of the parents, no matter which type of school you have planned for your child, an anthroposophic view of life?'; (3) 'Is the family's everyday life influenced by an anthroposophic view of life?'. Families answering 'anthroposophic school' to question 1 and 'yes' to questions 2 and 3, and also attending anthroposophic MCHCs were defined as being 'anthroposophic'. Families answering 'conventional' or any other non-anthroposophic type of school to question 1, 'no' to questions 2 and 3, and attending conventional MCHCs were defined as being 'non-anthroposophic'. Families with any other combination of answers were not included in this study.

The recruitment process and flow of participants in ALADDIN, described in detail elsewhere (Stenius *et al.*, 2011), resulted in 181 families eligible for this study, 82 families belonging to the anthroposophic and 99 families to the non-anthroposophic lifestyle group. Of these families we selected all mother–infant pairs that provided faecal samples from all or five of the six sampling occasions. The study was approved by the Research Ethical Committee at Huddinge University Hospital, Stockholm, Sweden, and written informed consent was obtained from all parents.

Preparation of samples for 454-pyrosequencing and taxonomic analysis of the sequence data

DNA was extracted from 250 mg stool samples using the MoBio Power Soil DNA Kit (Solana Beach, CA) according to the manufacturer's instructions with the only exception that the bead-beating step was performed 2 × 45 s, at level 5 on a FastPrep®-24 (MP Biomedicals, Solon, OH). The bacterial primers Bakt_341F (CCT ACGGGNGGCWGGAG) and Bakt_805R (GACTACHVG GGTATCTAATCC), complemented with 454 adapters and sample specific barcodes, were used to amplify the V3 and V4 regions of the 16S rRNA genes from the isolated DNA. Pre-processing of the amplicons was performed as described earlier (Roos *et al.*, 2013) and the prepared amplicon libraries were sequenced from the reverse primer direction at the Swedish Institute for Infectious Disease Control in Solna using the Roche/454 GS Titanium technology platform (Branford, CT). The obtained sequences from the 454-pyrosequencing analysis were processed and taxonomically classified as described previously (Herlemann *et al.*, 2011). In brief, after quality control, sequences were aligned and clustered using the

pyrosequencing pipeline at Ribosomal Database Project (RDP) with a conservative 5% dissimilarity to define operational taxonomic units (OTUs). The most abundant sequence from each OTU was selected as a representative sequence and was taxonomically classified by Basic Local Alignment Search Tool (BLAST), searching against a local BLAST database comprising 600 316 bacterial 16S rRNA gene sequences longer than 1200 bp with good Pintail scores from RDP v. 10.7. The OTU inherited the taxonomy (down to genus level) of the best scoring RDP hit fulfilling the criteria of $\geq$ 95% identity over an alignment of length $\geq$ 380 bp. Statistical evaluation of the data was performed on data taxonomically classified to genus level. Samples with fewer than 500 sequences were excluded from analysis. The sequences have been deposited in the sequence read archive (SRA) at the NCBI under accession number SRP049113.

Statistical analyses

The multivariate statistical software PAST version 2.17 (University of Oslo, Norway) was used for calculation of similarity indices, diversity and ordination (Hammer, 2012). The similarity between two samples was calculated using the Bray–Curtis similarity index. Biodiversity in a sample was measured using the Shannon index, which takes into account the number as well as the evenness of the genera in a sample. To avoid the influence of the number of sequences in the samples on the diversity-parameters the relative abundances in each sample were recalculated to correspond to a sample with 500 sequences, i.e. a least detectable relative abundance of 0.2%. All diversity calculations were also performed for a least detectable relative abundance of 0.1%, corresponding to 1000 sequences in a sample, but this did not alter the results (data not included).

Principal coordinate analysis (PCoA) based on abundance data from sequences classified to genus level was performed to determine clustering patterns among the subjects. To evaluate which factors were associated with the composition of the microbiota a permutational multivariate analysis of variance (PERMANOVA) based on Bray–Curtis distances and 1000 permutations was performed.

Differences in diversity and similarity indices were tested using the Mann–Whitney test or Kruskal–Wallis test and IBM SPSS STATISTICS 21 software (Chicago, IL). For differences in relative abundance of specific bacterial taxa we used Wilcoxon tests and linear regressions using the R statistical framework (R-project, 2013). To correct for multiple testing we performed 1000 permutations for each of the tests performed. For each permutation the abundance data for each taxon was randomly assigned to the explanatory variable. Based on the distributions of the *P*-values from the permuted tests we found that a false discovery rate (FDR) of 5.0% relates to an average *q*-value of 0.0501 (Storey, 2003).

Results

From the 128 mother–infant pairs included in this study a total of 708 stool samples were analysed, of which 43 (6.1%) had fewer than 500 sequences and were therefore excluded from further analysis. The remaining samples were: 116 from mothers before delivery; 116 from mothers after delivery; and 110, 101, 113 and 109 from the infants at ages 6 days, 3 weeks, 2 months and 6 months, respectively. The mean number of sequences per sample was 2670 (505–14 300). Of the 128 mother–infant pairs 55 (43%) were categorized as anthroposophic and 73 (57%) as non-anthroposophic. Among the families with anthroposophic lifestyle significantly more mothers were vegetarians, significantly more infants were born at home, and the infants were breastfed to a significantly larger extent than among families with a non-anthroposophic lifestyle (Table 1).

Gut microbiota composition in infants and mothers

The influence of age on gut microbiota is presented in Fig. 1. There was a clear difference between the infant and adult microbiota (Fig. 1a and c) with Firmicutes as the dominating phylum in the adult gut microbiota whereas Actinobacteria was the dominating phylum in infants. In addition, the abundance of Proteobacteria clearly differed between the young and adults with higher relative abundances in the infants (Fig. 1a).

The Shannon diversity index was calculated for each sample at the taxa level (Fig. 1). The mean Shannon diversity index in mothers (before birth) was 2.46 (95% CI 2.36–2.56) which was significantly higher than in infants. The mean Shannon diversity index at 6 months of age was 1.61 (1.49–1.72) which was significantly higher than 1.35 (1.23–1.47) at 2 months of age (Fig. 1c).

The Bray–Curtis index of similarity was calculated to investigate the similarity of microbiota composition between different samples. Comparisons were made within the family, demonstrating the stability of the microbiota within the mother, similarity between the mother and the samples from her infant, as well as between the consecutive samples of the infant. The mean similarity was highest between the two mother samples. The infant's microbiota was more similar to itself over time than to its mother's, but became more similar to its mother's with increasing age (Fig. 1d).

Table 1. Descriptive data of the mother–infant pairs in the two lifestyle groups

	Anthroposophic (n = 55) n (%)	Non-anthroposophic (n = 73) n (%)	P-value*
Living on a farm	9 (16.4)	11 (15.1)	0.84
Mother vegetarian	11 (20.0)	2 (2.7)	0.001
Antibiotics pregnancy†	8 (14.8)	11 (15.1)	0.97
Antibiotics delivery‡	5 (9.1)	6 (8.5)	0.90
Birth place home	24 (43.6)	0 (0)	< 0.001
Birth mode caesarean	7 (12.7)	12 (16.4)	0.56
Female sex	29 (52.7)	38 (52.1)	0.94
Milk formula 1st week†	7 (13.0)	17 (23.3)	0.14
Breastfeeding at 2 month			
Not	1 (1.8)	7 (9.6)	0.001
Partly	2 (3.6)	14 (19.2)	
Fully	52 (94.5)	52 (71.2)	
Breastfeeding at 6 month			
Not	3 (5.5)	17 (23.3)	< 0.001
Partly	30 (54.5)	47 (64.4)	
Fully	22 (40.0)	9 (12.3)	

**P*-values from chi-square test comparing anthroposophic with non-anthroposophic.
†Data missing from one anthroposophic family.
‡Data missing from two non-anthroposophic families.

Lifestyle exposures and gut microbiota composition

In the principal coordinate analysis no apparent clustering pattern was observed that distinguished the anthroposophic from the non-anthroposophic lifestyle groups (Supporting Information Fig. S1). This was confirmed using a PERMANOVA that revealed no significant influence of anthroposophic lifestyle on the global gut microbiota composition at the younger ages or among mothers. For 6-month-old infants, however, the P-value ($P = 0.04$) indicated an influence of the anthroposophic lifestyle on gut microbiota, but this was not statistically significant after correction for multiple testing (Table 2). We determined no significant influence of living on a farm, being vegetarian or having taken antibiotics during pregnancy on the mothers' gut microbiota. In infants, the influence of birth mode (caesarean vs. vaginal) had a large impact on the composition of the microbiota at the earliest ages (Table 2, Fig. S2). The difference declined with age and was not significant at 6 months of age ($P = 0.07$; Table 2). Breastfeeding significantly influenced the infant gut microbiota at 6 months of age, but not at 2 months of age (Table 2, Fig. S2). The infants' gut microbiota was not influenced by the following variables: if the mother was living on a farm; had a vegetarian diet; or had taken antibiotics during pregnancy. Neither did sex or the birthplace (home/hospital) have any significant impact on the infant gut microbiota composition.

We further tested which bacterial taxa were responsible for the differences in overall microbiota pattern as identified in the PERMANOVA. Table 3 depicts the mean relative abundances of the most abundant taxa in the children's gut microbiota, related to anthroposophy and the lifestyle exposures that were significant at any age in the PERMANOVA. Six-month-old children in families with an anthroposophic lifestyle had a significantly higher relative abundance of *Bifidobacterium* and lower relative abundance of *Bacteroides* and *Veillonella* (Table 3; Fig. S3). However, no significant association was observed between anthroposophic lifestyle and any of the most abundant taxa among mothers or the infants at the earlier ages.

At all investigated ages, the infants delivered by caesarean section had a lower relative abundance of *Bacteroides* (although not significantly at 6 months) and higher relative abundance of unclassified *Enterobacteriaceae* (significantly at all ages) and *Clostridium* (significantly at 3 weeks and 2 months) than the vaginally delivered infants. In addition, at 6 days and 3 weeks of age, these infants had a significantly lower abundance of *Bifidobacterium* and significantly higher abundance of *Haemophilus* and *Veillonella* compared with the vaginally delivered (Table 3; Fig. S4). Breastfed children had higher relative abundances of *Bifidobacterium* and *Streptococcus* but lower relative abundances of *Clostridiales*, *Clostridiaceae*1 and unclassified *Lachnospiraceae* at 6 months age (Table 3; Fig. S5).

The influence of lifestyle factors on the Shannon diversity index in the infant gut microbiota at the different ages is presented in Table 4. None of the investigated lifestyle factors, including anthroposophic lifestyle, signifi-

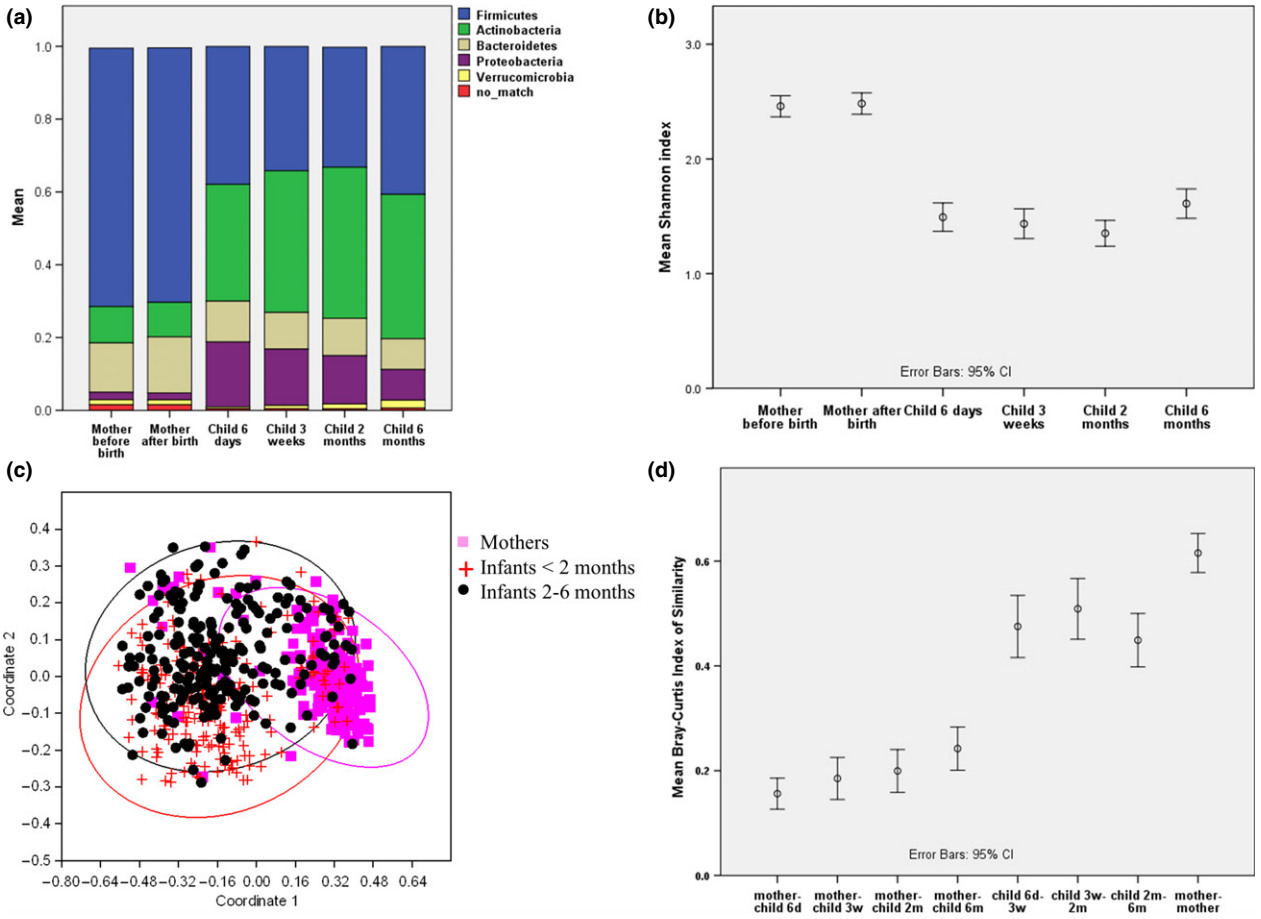

Fig. 1. (a) Mean relative abundances of phyla. (b) Mean Shannon diversity index for the different ages. (c) Principal coordinate analysis plot (Bray–Curtis distances). Ellipses represent 95% confidence interval. (d) Comparison of similarity between infants at the different ages and their respective mothers (1st to 4th circles from left), between two consecutive samples from the infants (5th to 7th circles) and between mother before and 2 months after delivery (8th circle). Circles represent mean Bray–Curtis index of similarity and error bars represent 95% confidence interval.

Table 2. *P*-values from PERMANOVA test to identify lifestyle factors that significantly influence the microbiota in infants and their mothers

	Mothers		Infants			
	Before delivery	After delivery	6 days	3 weeks	2 months	6 months
Lifestyle; anthroposophic/not	0.31	0.32	0.44	0.85	0.47	0.04
Living on a farm; yes/no	0.18	0.71	0.66	0.16	0.89	0.04
Mother vegetarian diet; yes/no	0.76	0.65	0.43	0.86	0.85	0.11
Antibiotics pregnancy; yes/no	0.59	0.20	–	–	–	–
Birth place; home/hospital†	–	–	0.68	0.75	0.67	0.08
Birth mode; vaginal/caesarean	–	–	0.0001*	0.0009*	0.02	0.07
Sex; boy/girl	–	–	0.95	0.91	0.43	0.91
Milk formula 1st week of life; yes/no	–	–	0.07	0.02	0.51	0.05
Breastfeeding 2 months; full/partly/not	–	–	–	–	0.28	0.003*
Breastfeeding 6 months; full/partly/not	–	–	–	–	–	0.002*

*Significant at alpha 0.05 after correcting for multiple testing using false discovery rates.
†Excludes those born with caesarian section.

Table 3. Mean relative abundance in per cent (and prevalence in per cent in brackets) of taxa at different ages in infants and their mothers, in relation to lifestyle exposures

	Lifestyle											
	A = Anthroposophic; NA = Non-Anthroposophic											
	Mothers				Infants							
Sample	Before		After birth		6 days		3 weeks		2 months		6 months	
Exposure group	A	NA	A	NA	A	NA	A	NA	A	NA	A	NA
n	50	66	51	65	45	65	44	57	49	64	47	62
Actinobacteria												
Bifidobacterium	7 (96)	10 (94)	7 (100)	9 (97)	35 (91)	26 (85)	39 (91)	36 (88)	37 (94)	42 (92)	45 (98)	33* (97)
Collinsella	1 (58)	1 (52)	1 (61)	1 (66)	1 (29)	1 (29)	1 (36)	1 (26)	2 (37)	1 (34)	1 (34)	1 (34)
Bacteroidetes												
Bacteroides	10 (90)	9 (86)	9 (88)	10 (94)	8 (67)	9 (62)	8 (52)	7 (44)	9 (57)	8 (67)	4 (64)	10*(65)
Parabacteroides	1 (58)	1 (59)	1 (63)	1 (63)	1 (29)	3 (28)	1 (23)	3 (23)	2 (29)	1 (22)	0 (21)	0 (26)
Proteobacteria												
Unclassified_	0 (6)	0 (6)	1 (4)	0 (6)	3 (22)	4 (19)	5 (36)	3 (35)	2 (37)	2 (38)	1 (28)	1 (32)
Enterobacteriaceae												
Enterobacteriaceae	1 (26)	1 (29)	0 (20)	1 (25)	8 (47)	12 (51)	7 (64)	9 (67)	6 (71)	11 (73)	4 (79)	8 (82)
Haemophilus	0 (8)	0 (11)	0 (6)	0 (11)	0 (16)	2 (32)	1 (27)	2 (37)	1 (25)	1 (27)	1 (26)	0 (29)
Firmicutes												
Clostridiales	18 (98)	17 (94)	20 (100)	16 (97)	7 (71)	7 (62)	4 (43)	8 (60)	7 (51)	8 (56)	12 (79)	13 (81)
*Clostridiaceae*1	0 (24)	1 (33)	0 (24)	1 (29)	1 (16)	3 (29)	5 (25)	2 (23)	2 (22)	1 (30)	1 (26)	2 (37)
Clostridium	1 (32)	1 (41)	0 (31)	1 (37)	1 (16)	2 (20)	1 (30)	1 (19)	7 (29)	4 (28)	4 (47)	1 (48)
Unclassified_	8 (96)	7 (89)	6 (92)	8 (92)	2 (38)	1 (31)	1 (25)	1 (28)	1 (14)	1 (23)	1 (38)	1 (26)
Ruminococcaceae												
Unclassified_	5 (98)	5 (89)	5 (100)	4*(92)	2 (33)	2 (26)	1 (23)	1 (32)	1 (31)	1 (31)	1 (45)	2 (55)
Lachnospiraceae												
Veillonella	1 (26)	0 (21)	0 (12)	0 (17)	4 (42)	4 (37)	6 (71)	6 (60)	8 (65)	5 (75)	4 (66)	8*(79)
Streptococcaceae	0 (28)	0 (29)	0 (22)	0 (20)	2 (73)	4 (74)	3 (77)	3 (70)	1 (43)	1 (58)	1 (38)	0 (40)
Streptococcus	0 (24)	0 (20)	0 (14)	0 (20)	2 (64)	2 (71)	3 (68)	2 (70)	2 (61)	1 (66)	2 (77)	1 (53)
Verrucomicrobia												
Akkermansia	2 (50)	1 (39)	1 (43)	2 (51)	1 (20)	0 (11)	0 (11)	1 (9)	2 (12)	1 (13)	2 (19)	2 (19)

Only taxa with mean relative abundance > 1% in child samples are displayed. Wilcoxon tests or regression was used to calculate *P*-values for differences between exposure groups at the various ages. False discovery rate values (q-values) were calculated to correct for multiple testing. Abundances that are statistically different between exposure groups at a significance level of 0.05 (and $P < q$) are marked with an asterisk (*).

cantly influenced the Shannon diversity index in the infant gut microbiota after correcting for multiple testing. However, there was an obvious trend for an inverse relationship between breastfeeding and diversity at both 2 and 6 months of age. There were no signs of reduced diversity among the children delivered with caesarean section in our data. All diversity calculations were also performed both at OTU-level and by using the Simpson diversity index, with similar results for lifestyle influence (data not included).

Discussion

A large impact of lifestyle on gut microbiota has been reported in studies comparing children from very different environments, such as rural Malawi vs. metropolitan USA (Yatsunenko *et al.*, 2012), and rural Africa vs. urban Italy (De Filippo *et al.*, 2010). In this prospective and population-based study we compared the gut microbiota of mothers and infants from the same geographical area (Järna, outside Stockholm, Sweden) who were exposed to two different lifestyles, namely an anthroposophic lifestyle, which has been associated with reduced allergy risk among children, and a conventional Swedish lifestyle, which has a high prevalence of allergic disease, similar to that found in many other western countries. We could not demonstrate a significant influence of the anthroposophic lifestyle on the overall gut microbiota pattern in either mothers or infants. However, when specific taxa were compared between 6-month-old infants in anthroposophic families and controls, we recorded a significantly higher relative abundance of *Bifidobacterium* and a significantly lower abundance of

Birth mode								Breastfeeding 2 month								
V = Vaginal; C = Caesarean								Fully (+); Partly (+/–);Not (–)						Breastf 6 months		
6 days		3 weeks		2 months		6 months		2 months			6 months			6 months		
V	C	V	C	V	C	V	C	+	+/-	-	+	+/-	-	+	+/-	-
94	16	87	14	95	18	91	18	94	14	5	88	15	6	22	70	17
34 (93)	4*(56)	40 (91)	18*(79)	41 (95)	34 (83)	40 (97)	30 (100)	42 (95)	36 (93)	13 (60)	42 (98)	23 (93)	12*(100)	51 (100)	39 (97)	18*(94)
1 (33)	0 (6)	1 (33)	1 (14)	2 (40)	0 (11)	2 (37)	1 (17)	2 (38)	0 (14)	3 (40)	1 (34)	2 (33)	1 (33)	1 (23)	1 (37)	2 (35)
10 (67)	0*(44)	9 (53)	0*(14)	10 (70)	1*(28)	8 (67)	4 (50)	9 (62)	7 (71)	9 (60)	7 (66)	9 (67)	7 (33)	7 (73)	8 (63)	6 (59)
3 (32)	0 (6)	3 (26)	0 (0)	1 (30)	0 (0)	0 (25)	0 (17)	1 (25)	0 (21)	0 (40)	0 (23)	0 (33)	0 (17)	1 (32)	0 (23)	0 (18)
2 (16)	12*(44)	2 (31)	11*(64)	1 (30)	5*(78)	0 (24)	2*(61)	2 (31)	1 (71)	3 (60)	1 (24)	1 (53)	3 (67)	1 (23)	0 (24)	1 (65)
10 (48)	10 (56)	8 (63)	6 (79)	9 (72)	11 (78)	6 (81)	4 (78)	8 (69)	14 (86)	18 (100)	5 (81)	7 (87)	13 (67)	6 (82)	5 (81)	9 (77)
1 (20)	4*(56)	1 (28)	6*(64)	1 (25)	2 (28)	1 (26)	0 (33)	1 (28)	0 (14)	0 (20)	1 (26)	0 (33)	0 (33)	1 (23)	1 (30)	0 (24)
7 (66)	6 (63)	6 (53)	10 (50)	8 (53)	7 (61)	11 (78)	20*(89)	7 (52)	12 (57)	11 (80)	10 (76)	25 (93)	21*(100)	6 (82)	12 (77)	22 (88)
1 (19)	10*(50)	3 (22)	4 (36)	2 (27)	0 (22)	1 (29)	4 (50)	2 (23)	1 (29)	1 (80)	2 (30)	3 (47)	0 (33)	0 (23)	1 (30)	8*(53)
1 (14)	4 (44)	1 (22)	4*(36)	4 (25)	16*(44)	2 (43)	4 (72)	5 (28)	11 (29)	1 (40)	3 (48)	1 (47)	0 (50)	1 (32)	3 (49)	3 (65)
1 (37)	1 (13)	1 (29)	1 (14)	1 (19)	1 (22)	1 (32)	1 (28)	1 (19)	1 (21)	0 (20)	1 (31)	1 (27)	2 (50)	0 (27)	1 (30)	1 (41)
2 (30)	1 (25)	1 (28)	1 (29)	1 (34)	1 (17)	1 (48)	1 (61)	1 (29)	0 (36)	1 (60)	1 (42)	1 (87)	4*(83)	1 (23)	1 (53)	2 (77)
2 (33)	11*(75)	4 (63)	17*(71)	6 (72)	5 (67)	7 (73)	5 (78)	6 (69)	5 (86)	17 (60)	6 (72)	7 (87)	8 (67)	5 (77)	7 (70)	7 (82)
3 (75)	2 (69)	3 (75)	1 (64)	1 (50)	1 (61)	1 (40)	1 (39)	1 (48)	1 (71)	2 (60)	1 (39)	0 (47)	1 (33)	0 (32)	1 (43)	0 (35)
2 (67)	3 (75)	3 (70)	1 (64)	2 (62)	2 (72)	1 (63)	1 (67)	2 (63)	1 (64)	3 (80)	1 (67)	0 (47)	1 (50)	3 (73)	1 (63)	1*(53)
0 (16)	0 (6)	1 (10)	0 (7)	1 (13)	1 (11)	2 (19)	3 (22)	2 (13)	0 (7)	0 (20)	3 (23)	0 (7)	0 (0)	3 (13)	2 (23)	0 (12)

Bacteroides and *Veillonella* in infants from anthroposophic families. Furthermore, our data demonstrated that mode of delivery and breastfeeding had a significant influence on infant gut microbiota composition.

Mode of delivery is known to influence the infant gut microbiota. In accordance with other studies (Gronlund *et al.*, 1999; Penders *et al.*, 2006; Fallani *et al.*, 2010; Azad *et al.*, 2013a, b; Jakobsson *et al.*, 2014), we observed that the children delivered by caesarean section had a reduced relative abundance of *Bacteroides* at all investigated time points. A higher abundance of unclassified *Enterobacteriaceae*, *Veillonella* (Jakobsson *et al.*, 2014) and *Haemophilus* (Azad *et al.*, 2013a, b) has also been reported in earlier studies. Another important difference associated with birth mode was that children delivered by caesarean section had a dramatically lower abundance of *Bifidobacterium* at 6 days and 3 weeks of age, but no longer at 2 months of age. *Bifidobacterium* was already the dominant taxon in the infant gut microbiota at age 6 days. A similar influence of birth mode on *Bifidobacterium* has been demonstrated in other studies (Gronlund *et al.*, 1999; Penders *et al.*, 2006; Biasucci *et al.*, 2010; Tannock *et al.*, 2013), and support for a mother-to-child transmission of bifidobacterial strains was reported in a recent study (Makino *et al.*, 2013).

Bifidobacteria can also be transferred via the mother's milk. Human milk contains bifidobacteria (Jeurink *et al.*, 2013) and moreover contains a complex mixture of oligosaccharides that can favour growth of the bifidobacterial population in the infant gut (Zivkovic *et al.*, 2011). Breastfeeding subsequently contributed strongly to a higher abundance of *Bifidobacterium*, which is in agreement with what has been previously determined (Fallani *et al.*, 2011; Tannock *et al.*, 2013). Our data demon-

Table 4. Median Shannon (SI) diversity index in stool samples from infants and their mothers at the different ages, according to different lifestyle factors

	Children age								Mother			
	6 days		3 weeks		2 months		6 months		Before		After birth	
	n	SI	*n*	SI	*n*	SI	*n*	SI	*n*	SI	*n*	SI
Lifestyle												
Antroposophic	45	1.45	44	1.43	49	1.31	47	1.35	50	2.59	51	2.58
Not	65	1.47	57	1.41	64	1.33	62	1.64	66	2.59	65	2.60
Living on a farm												
No	91	1.45	84	1.41	94	1.34	91	1.63	98	2.60	99	2.59
Yes	19	1.49	17	1.53	19	1.30	18	1.25	18	2.48	17	2.58
Mother vegetarian												
No	99	1.47	90	1.41	100	1.33	97	***1.63***	103	2.60	104	2.58
Yes	11	1.45	11	1.50	13	1.31	12	***1.10***	13	2.58	12	2.73
Antibiotics pregnancy												
No	97	1.39	87	1.43	98	1.30	95	1.60	100	2.60	99	2.59
Yes	13	1.81	14	1.26	15	1.34	14	1.53	16	2.57	16	2.53
Birthplace												
Hospital*	73	1.38	68	1.40	75	1.32	70	1.52		–		–
Home	21	1.35	19	1.39	20	1.40	21	1.36		–		–
Birthmode												
Vaginal	94	1.37	87	1.39	95	1.33	91	1.52		–		–
Caesarean	16	1.59	14	1.62	18	1.24	18	1.70		–		–
Sex												
Boy	51	1.48	44	1.41	53	1.30	53	1.49		–		–
Girl	59	1.40	57	1.41	60	1.36	56	1.62		–		–
Milk formula 1st week												
Yes	27	1.33	25	1.35	28	1.29	29	1.78		–		–
No	89	1.45	80	1.39	89	1.30	87	1.49		–		–
Breastfeeding 2 months												
Exclusive	–	–	–	–	94	1.30	88	***1.39***		–		–
Partly	–	–	–	–	14	1.58	15	***1.84***		–		–
Not	–	–	–	–	5	1.66	6	***2.14***		–		–
Breastfeeding 6 months												
Exclusive	–	–	–	–	–	–	25	***1.32***		–		–
Partly	–	–	–	–	–	–	67	***1.60***		–		–
Not	–	–	–	–	–	–	17	***1.94***		–		–
All samples	110	1.49	101	1.44	113	1.35	109	1.61	116	2.46	116	2.48

Median Shannon Weiner diversity index in bold and italic style where *P* for difference between exposure groups is < 0.05 (calculated with Mann–Whitney U-test for two exposure groups and Kruskal–Wallis test for three). None of the differences were significant after adjusting for multiple testing.

*Vaginally delivery only.

strated that infants exclusively breastfed at 6 months of age had the highest abundances, partly breastfed infants had intermediate abundances, and infants who were not breastfed had the lowest abundances of *Bifidobacterium*. Even though breastfeeding might be delayed among mothers who have gone through caesarean section, the proportion of infants who had been given formula during the first week of life was rather low and similar among those delivered by caesarean section (21%) and those delivered vaginally (19%). Thus the difference in relative abundance of *Bifidobacterium* between the two groups of birth mode cannot be explained by differences in breastfeeding.

In contrast to the large impact of infant feeding on the infants' gut microbiota, we found no significant influence of vegetarian diet on the mothers' gut microbiota. However, this study was not designed to investigate the impact of vegetarian diet, which could be one reason why no differences were apparent between vegetarian and non-vegetarian mothers.

At 6 months of age, 94.5% of the anthroposophic and 76.7% of the non-anthroposophic children were still being breastfed. Breastfeeding has also been associated with a reduced abundance of *Bacteroides* (Penders *et al.*, 2006; Fallani *et al.*, 2010) and could therefore explain both the higher relative abundance of *Bifidobacterium* and the lower

relative abundance of *Bacteroides* among the anthroposophic children at 6 months of age. However, in the ALADDIN birth cohort, of whom these children are a subsample, the risk of allergic sensitization before 2 years of age among children with anthroposophic lifestyle was as low as one-quarter of that among in those the non-anthroposophic group, even after adjusting for breastfeeding (Stenius *et al.*, 2011). It is therefore unlikely that the different relative abundances of *Bifidobacterium* and *Bacteroides* are important for the allergy-protective effect of this lifestyle.

The Shannon index of diversity is widely used as a measure of gut microbiota diversity, and our results are consistent with the literature in that gut microbiota diversity increases with age (Forno *et al.*, 2008; Jakobsson *et al.*, 2014) and that breastfeeding delays this increase (Azad *et al.*, 2013a, b). However, we found no association between caesarean section and Shannon diversity index, as has been indicated in other studies (Azad *et al.*, 2013a, b; Jakobsson *et al.*, 2014). Overall diversity might be a too simplified a measure of gut microbiota when studying its association with exposure factors and health outcome such as allergic disease (Azad *et al.*, 2013a, b). This could explain our failure to confirm the higher gut microbiota diversity among anthroposophic children reported in another study (Dicksved *et al.*, 2007). That study, as well as an earlier culture-based study (Alm *et al.*, 2002), indicated a different composition of the lactic acid bacteria group among anthroposophic children. The present study did not focus on the lactobacilli population, however, and the depth of sequencing did not allow us to study the abundance and diversity of less dominant taxa such as lactobacilli, although this could be an important focus in future studies.

The strengths of this study are, in addition to its prospective, population-based design, the relatively large number of participants, the frequent collection of stool samples at very young age and the reliable information on lifestyle exposures collected during repeated interviews with parents. The depth of the sequencing did allow detection of the dominant microbiota, but the species prevalent at lower abundances could not be detected. Furthermore, because many studies of gut microbiota and allergic disease indicate associations at a species or strain level it is possible that such associations were undetected in this analysis (Björksten *et al.*, 1999; Johansson *et al.*, 2011). The development of sequencing methods has increased sequencing capacity dramatically, enabling detection of low abundant rare species. Moreover, the increased sequencing capacity has also facilitated use of metagenomic approaches to study the gut microbiome which avoid the biases inherited in PCR-based approaches.

In summary, we did not observe any strong influence of anthroposophic lifestyle on the global microbiota pattern, either in infants or in their mothers, despite large differences in certain lifestyle exposures. Of the investigated, more specific, lifestyle factors, both birth mode (caesarean vs. vaginal) and breastfeeding had a major impact on the infant gut microbiota and strongly affected the *Bifidobacterium* population. However, other factors such as mother's diet, living on a farm during pregnancy, mother's exposure to antibiotics during pregnancy or home birth did not imprint any detectable influences on either the mothers' or the infants' global gut microbiota profile. Additionally, in 6-month-old infants we determined a significantly higher abundance of the *Bifidobacterium* population when anthroposophic infants were compared with the controls. This difference was likely influenced by the prolonged breastfeeding associated with the anthroposophic lifestyle. Further analyses that include more samples, a methodology with better taxonomic resolution, and deeper coverage of the gut microbiota would be necessary to disentangle whether the allergy-protective effect of this lifestyle is mediated by the gut microbiota.

Acknowledgements

The authors thank the families participating in the ALADDIN study for their trust and contribution and the ALADDIN team for their involvement in this work, especially nurse and coordinator Margareta Eriksson and biomedical analyst Monica Nordlund. The skilled technical assistance of Elin Lundin is also gratefully acknowledged, and Prof. Robert A. Harris for linguistic advice. This study was funded and supported by grants from the Centre for Allergy Research and KID at Karolinska Institutet, the 'Mjölkdroppen' Society, the Swedish Asthma and Allergy Research Association, the Swedish Research Council, Swedish Research Council for Working Life and Social Research, the Swedish Society of Medicine, the Cancer- and Allergy Fund and the Ekhaga Foundation, the 'Frimurare Barnhuset' in Stockholm, and the Gyllenberg-, Hesselman-, Samariten and Vårdal Foundations.

References

Abrahamsson TR, Jakobsson HE, Andersson AF, Björksten B, Engstrand L & Jenmalm MC (2012) Low diversity of the gut microbiota in infants with atopic eczema. *J Allergy Clin Immunol* **129**: 434–440, 440 e431–432.

Alm JS, Swartz J, Lilja G, Scheynius A & Pershagen G (1999) Atopy in children of families with an anthroposophic lifestyle. *Lancet* **353**: 1485–1488.

Alm JS, Swartz J, Björksten B *et al.* (2002) An anthroposophic lifestyle and intestinal microflora in infancy. *Pediatr Allergy Immunol* **13**: 402–411.

Andersson AF, Lindberg M, Jakobsson H, Backhed F, Nyren P & Engstrand L (2008) Comparative analysis of human gut microbiota by barcoded pyrosequencing. *PLoS ONE* **3**: e2836.

Azad MB, Konya T, Maughan H, Guttman DS, Field CJ, Chari RS, Sears MR, Becker AB, Scott JA & Kozyrskyj AL (2013a) Gut microbiota of healthy Canadian infants: profiles by mode of delivery and infant diet at 4 months. *CMAJ* **185**: 385–394.

Azad MB, Konya T, Maughan H, Guttman DS, Field CJ, Sears MR, Becker AB, Scott JA & Kozyrskyj AL (2013b) Infant gut microbiota and the hygiene hypothesis of allergic disease: impact of household pets and siblings on microbiota composition and diversity. *Allergy Asthma Clin Immunol* **9**: 15.

Biasucci G, Rubini M, Riboni S, Morelli L, Bessi E & Retetangos C (2010) Mode of delivery affects the bacterial community in the newborn gut. *Early Hum Dev* **86**(suppl 1): 13–15.

Björksten B, Naaber P, Sepp E & Mikelsaar M (1999) The intestinal microflora in allergic Estonian and Swedish 2-year-old children. *Clin Exp Allergy* **29**: 342–346.

De Filippo C, Cavalieri D, Di Paola M, Ramazzotti M, Poullet JB, Massart S, Collini S, Pieraccini G & Lionetti P (2010) Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. *P Natl Acad Sci USA* **107**: 14691–14696.

Dicksved J, Flöistrup H, Bergström A *et al.* (2007) Molecular fingerprinting of the fecal microbiota of children raised according to different lifestyles. *Appl Environ Microbiol* **73**: 2284–2289.

Dicksved J, Halfvarson J, Rosenquist M, Jarnerot G, Tysk C, Apajalahti J, Engstrand L & Jansson JK (2008) Molecular analysis of the gut microbiota of identical twins with Crohn's disease. *ISME J* **2**: 716–727.

Dominguez-Bello MG, Costello EK, Contreras M, Magris M, Hidalgo G, Fierer N & Knight R (2010) Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns. *P Natl Acad Sci USA* **107**: 11971–11975.

Dominguez-Bello MG, Blaser MJ, Ley RE & Knight R (2011) Development of the human gastrointestinal microbiota and insights from high-throughput sequencing. *Gastroenterology* **140**: 1713–1719.

Fallani M, Young D, Scott J *et al.* (2010) Intestinal microbiota of 6-week-old infants across Europe: geographic influence beyond delivery mode, breast-feeding, and antibiotics. *J Pediatr Gastroenterol Nutr* **51**: 77–84.

Fallani M, Amarri S, Uusijarvi A *et al.* (2011) Determinants of the human infant intestinal microbiota after the introduction of first complementary foods in infant samples from five European centres. *Microbiology* **157**(Pt 5): 1385–1392.

Flöistrup H, Swartz J, Bergström A *et al.* (2006) Allergic disease and sensitization in Steiner school children. *J Allergy Clin Immunol* **117**: 59–66.

Forno E, Onderdonk AB, McCracken J *et al.* (2008) Diversity of the gut microbiota and eczema in early life. *Clin Mol Allergy* **6**: 11.

Gronlund MM, Lehtonen OP, Eerola E & Kero P (1999) Fecal microflora in healthy infants born by different methods of delivery: permanent changes in intestinal flora after cesarean delivery. *J Pediatr Gastroenterol Nutr* **28**: 19–25.

Hammer Ø (2012) PAST PAleontological STatistics Version 2.17 Reference Manual. From http://www.nhm2.uio.no/norlex/past/pastmanual.pdf

Herlemann DP, Labrenz M, Jurgens K, Bertilsson S, Waniek JJ & Andersson AF (2011) Transitions in bacterial communities along the 2000 km salinity gradient of the Baltic Sea. *ISME J* **5**: 1571–1579.

Jakobsson HE, Abrahamsson TR, Jenmalm MC, Harris K, Quince C, Jernberg C, Bjorksten B, Engstrand L & Andersson AF (2014) Decreased gut microbiota diversity, delayed *Bacteroidetes* colonisation and reduced Th1 responses in infants delivered by caesarean section. *Gut* **63**: 559–566.

Jeurink PV, van Bergenhenegouwen J, Jimenez E, Knippels LM, Fernandez L, Garssen J, Knol J, Rodriguez JM & Martin R (2013) Human milk: a source of more life than we imagine. *Benef Microbes* **4**: 17–30.

Johansson MA, Sjogren YM, Persson JO, Nilsson C & Sverremark-Ekstrom E (2011) Early colonization with a group of Lactobacilli decreases the risk for allergy at five years of age despite allergic heredity. *PLoS ONE* **6**: e23031.

Makino H, Kushiro A, Ishikawa E *et al.* (2013) Mother-to-infant transmission of intestinal bifidobacterial strains has an impact on the early development of vaginally delivered infant's microbiota. *PLoS ONE* **8**: e78331.

Penders J, Thijs C, Vink C, Stelma FF, Snijders B, Kummeling I, van den Brandt PA & Stobberingh EE (2006) Factors influencing the composition of the intestinal microbiota in early infancy. *Pediatrics* **118**: 511–521.

Penders J, Stobberingh EE, van den Brandt PA & Thijs C (2007) The role of the intestinal microbiota in the development of atopic disorders. *Allergy* **62**: 1223–1236.

Roos S, Dicksved J, Tarasco V, Locatelli E, Ricceri F, Grandin U & Savino F (2013) 454 pyrosequencing analysis on faecal samples from a randomized DBPC trial of colicky infants treated with *Lactobacillus reuteri* DSM 17938. *PLoS ONE* **8**: e56710.

R-project (2013) R: A Language and Environment for Statistical Computing. From http://www.R-project.org

Stenius F, Swartz J, Lindblad F, Pershagen G, Scheynius A, Alm J & Theorell T (2010) Low salivary cortisol levels in infants of families with an anthroposophic lifestyle. *Psychoneuroendocrinology* **35**: 1431–1437.

Stenius F, Swartz J, Lilja G, Borres M, Bottai M, Pershagen G, Scheynius A & Alm J (2011) Lifestyle factors and sensitization in children – the ALADDIN birth cohort. *Allergy* **66**: 1330–1338.

Storey JD (2003) The positive false dicovery rate: a Bayesian interpretation and the q-value. *Ann Stat* **31**: 2013–2035.

Tannock GW, Lawley B, Munro K *et al.* (2013) Comparison of the compositions of the stool microbiotas of infants fed goat milk formula, cow milk-based formula, or breast milk. *Appl Environ Microbiol* **79**: 3040–3048.

Wang M, Karlsson C, Olsson C *et al.* (2008) Reduced diversity in the early fecal microbiota of infants with atopic eczema. *J Allergy Clin Immunol* **121**: 129–134.

Yatsunenko T, Rey FE, Manary MJ *et al.* (2012) Human gut microbiome viewed across age and geography. *Nature* **486**: 222–227.

Zivkovic AM, German JB, Lebrilla CB & Mills DA (2011) Human milk glycobiome and its impact on the infant gastrointestinal microbiota. *P Natl Acad Sci USA* **108**(suppl 1): 4653–4658.

Zoetendal EG, Akkermans ADL, Akkermans-van Vliet WM, de Visser JAGM & de Vos WM (2001) The host genotype affects the bacterial community in the human gastronintestinal tract. *Microb Ecol Health Dis* **13**: 129–134.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. PCA-plots of faecal samples from infants at the different ages. Circles: anthroposophic; triangles: non-anthroposophic.

Fig. S2. PCA-plots of faecal samples from infants at different ages coloured according to delivery-mode and breastfeeding.

Fig. S3. Box plots illustrating the distribution of relative abundances for taxa that differ significantly between lifestyles at any of the child ages.

Fig. S4. Box plots illustrating the distribution of relative abundances for taxa that differ significantly according to birth mode.

Fig. S5. Box plots illustrating the distribution of relative abundances for taxa that differ significantly according to the level of breastfeeding at 2 months (BRF2) and 6 months (BRF6).