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Low diversity of the gut microbiota in infants developing atopic eczema

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ABSTRACT

Background: It is debated whether a low total diversity of the gut microbiota in early childhood is more important than altered prevalence of particular bacterial species for the increasing incidence of allergic disease. The advent of powerful, cultivation-free, molecular methods makes it possible to characterize the total microbiome down to the genus level in large cohorts.

Objective: To assess microbial diversity and characterize the dominant bacteria in stool during the first year of life in relation to atopic eczema development.

Methods: The microbial diversity and composition was analyzed with barcoded 16S rDNA 454-pyrosequencing in stool samples at one week, one month and 12 months of age in 20 infants developing IgE-associated eczema and 20 infants without any allergic manifestation until two years of age. (ClinicalTrials.gov ID NCT01285830)

Results: Infants who developed IgE-associated eczema had a lower diversity of the total microbiota at one month ($p=0.004$) and lower diversity of the bacterial phyla Bacteriodetes and the genus *Bacteroides* at one month ($p=0.02$ and $p=0.01$) and Proteobacteria at 12 months of age ($p=0.02$). The microbiota was less uniform at one month than 12 months of age, with a high inter-individual variability. At 12 months, when the microbiota had stabilized, Proteobacteria, comprising gram negatives, were more abundant in infants without allergic manifestation (Edge R test $p=0.008$, $q=0.02$).

Conclusion: Low intestinal microbial diversity during the first month of life was associated with subsequent atopic eczema.

Key message: Low microbial diversity early in life is associated with increased risk for allergic disease.

Capsule summary

With a novel powerful non-cultivation based method, infants who developed atopic eczema were shown to have a low intestinal microbial diversity during the first month of life, in particular low diversity of Bacteroidetes and Protebacteria.

Key words

Allergic disease; *Bacteroides;* diversity; eczema; hygiene hypothesis; infant; microbiota; molecular microbiology; pyrosequencing; *Sutterella*

Abbreviations

BLAST: Basic Local Alignment Search Tool

CV: Coefficient of variance

DGGE: Denaturating gradient gel electrophoresis

Edge R: Empirical analysis of digital gene expression in R

FISH: Fluorescent in situ hybridization

LPS: Lipopolysaccharides

OTU: Operational Taxonomic Unit

RDP: Ribosomal Database Project

SPT: skin prick test

T-RFLP: Terminal restriction fragment length polymorphism

INTRODUCTION

It is debated whether low diversity of the gut microbiota in infancy is more important than the prevalence of specific bacterial taxa when trying to explain why the prevalence of allergic disease is increasing in affluent countries. Initially, several studies employing conventional cultivation or fluorescent in situ hybridization (FISH) reported differences in the intestinal microbiota at a species level between allergic and non-allergic children. [1-3] Allergic infants were colonized less often with *Bacteroides and* bifidobacteria, [1, 2] more often with *Staphylococcus aureus,* [2] and they had lower ratio of bifidobacteria to clostridia. [3] However, there have been contradictory results in more recent studies. Two large European prospective studies did not confirm any relationship with any particular bacterial group. [4, 5]

As an alternative explanation, it has been suggested that low diversity of intestinal microbiota would explain the increase of allergic disease in affluent societies. [6, 7] The underlying rationale is that the gut immune system reacts to exposure to new bacterial antigens and repeated exposure would enhance the development of immune regulation. Although this theory emerged more than a decade ago,[8] there are still only few studies relating the diversity with allergy, likely due to methodology limitations. In three studies employing molecular techniques, terminal restriction fragment length polymorphism (T-RFLP) [6] and denaturating gradient gel electrophoresis (DGGE)[9, 10], infants developing sensitization [10] or eczema [6 9] were reported to have fewer peaks/bands than healthy ones. Yet, no specific microbes were identified with these molecular methods. Furthermore, the sensitivity of the methods appears to be low, since the median number of peaks/bands was much lower than the expected number of bacterial species. [6, 9, 10]

A new generation of powerful non-cultivation microbiology methods has now made it possible to analyze the total microbiota down to the genus level, even in large cohorts. [11, 12]

Previously uncultivated bacteria can now be detected, and there is no need to decide what bacteria to analyze in advance. Thus the assessment can be made unprejudiced. This will allow more comprehensive knowledge of the intestinal microbiota and its impacts on the immune system. We have employed barcoded 16S rRNA 454-pyrosequencing [13] to assess the microbial diversity and characterize the dominant bacteria in stool during the first year of life in infants who either developed atopic eczema or did not have any allergic manifestation up to two years of age.

METHODS

Study design

The infants included in this study were part of a larger study in South Eastern Sweden between 2001 and 2005, evaluating allergy prevention with the probiotic *Lactobacillus reuteri* ATCC 55730. [14] In this study the infant received probiotics or placebo daily from day 1-3 until 12 months of age. Clinical follow-ups were done at 1, 3, 6, 12 and 24 months of age and telephone interviews at 2, 4, 5, 8, 10 and 18 months. A questionnaire was completed on each occasion. Stool samples were collected from the infants at age 5-7 days and at one month and 12 months of age. The samples were immediately frozen at -20°C following collection and later stored at -70°C. Among the 188 infants completing the original study and from which stool samples were available from all three sampling occasions, 20 infants with atopic eczema and 20 without any allergic manifestation were randomly selected to this study.

There were no differences regarding potential confounders such as sex, birth order, caesarean section, family history of allergic disease, breastfeeding, antibiotics and probiotic supplementation between the infants with and without atopic eczema (Table I). Children admitted to the neonatal ward during the first week of life were excluded from the original study. All infants were breastfed for at least one month, and no infant received antibiotics before one month of age. An informed consent was obtained from both parents before inclusion. The Regional Ethics Committee for Human Research at Linköping University approved the study. The study is registered at ClinicalTrials.gov (ID NCT01285830).

Diagnostic criteria of atopic eczema

Eczema was defined as a pruritic, chronic or chronically relapsing non-infectious dermatitis with typical features and distribution. [14] The diagnosis atopic eczema required that the infant

with eczema also was sensitized. [15] Infants were regarded as sensitized if they had at least one positive SPT and/or detectable circulating allergen specific IgE antibodies. Skin prick tests were done on the volar aspects of the forearm with egg white, fresh skimmed cow milk (lipid concentration 0.5%) and standardised cat, birch and timothy extracts (Soluprick®, ALK, Hørsholm, Denmark) at 6, 12 and 24 months of age. Histamine hydrochloride (10 mg/ml) was used as positive and albumin diluents as negative control. The test was regarded as positive if the mean diameter of the wheal was >3mm. Circulating IgE antibodies to egg white and cow's milk were analysed at 6, 12, and 24 months of age in venous blood (UniCap® Pharmacia CAP System™, Pharmacia Diagnostics, Uppsala, Sweden). The cut off level was 0.35 kU/L, according to the protocol of the manufacturer. In addition, circulating IgE to a mixture of food allergens, including egg white, cow's milk, cod, wheat, peanut and soy bean, was analysed at 6, 12 and 24 months of age (UniCap® Pharmacia CAP System™, fx5, Pharmacia Diagnostics).

DNA extraction, 16S rRNA gene amplification, and sequencing

Extraction of bacterial DNA from the fecal samples and the 16S rRNA gene amplification was made according to a previous publication [13] with the following modifications; the primer pair used, targeting the variable regions 3 and 4 of the 16S rRNA gene, were 341f 5´CCTACGGGNGGCWGCAG with adaptor B and 805r 5´GACTACHVGGGTATCTAATCC with adaptor A [16] and sample-specific sequence barcodes consisting of five nucleotides. The barcodes contained no homopolymers and a pair of barcodes differed in at least two positions. A negative PCR reaction without template was also included for all primer pairs in each run. The PCR was run for 25 cycles. The PCR-products with proximal lengths of 450 bp were purified with AMPure beads (Becton Dickinson, Franklin, USA) using a Magnet Particle Separator (Invitrogen, Carlsbad, CA,

172 USA). The concentrations were measured by Qubit fluorometer (Invitrogen) CA), the quality
173 was assessed on a Bioanalyzer 2100 (Agilent, Santa Clara, USA), and the samples were
174 pooled together and amplified in PCR-mixture-in-oil emulsions and sequenced on different
175 lanes of a 2-lane PicoTiterPlate on a Genome Sequencer FLX system (Roche, Basel,
176 Switzerland) at the Royal Institute of Technology (KTH) in Stockholm.
177
178 **Sequence processing and taxonomic classification**
179 Sequence processing was carried out with the AmpliconNoise software package [17] correcting
180 for errors introduced in the PCR and pyrosequencing as well as removing chimeric sequences.
181 Also, reads lacking a correct primer and/or having less than 360 successful pyrosequencing
182 flows were removed. [17] Denoised sequences were trimmed to 198 bp after primer and barcode
183 removal and clustered by complete linkage clustering into operational taxonomic units
184 (OTUs) at the 97% similarity level using AmpliconNoise. [17] Each denoised sequence, as well
185 as the most abundant sequence for each OTU, was BLAST searched with default parameters
186 against a local BLAST database comprising 836.814 near full-length bacterial 16S rRNA
187 gene sequences from the Ribosomal Database Project (RDP) v. 10.10. [18] The sequences
188 inherited the taxonomic annotation (down to genus level) of the best scoring RDP hit
189 fulfilling the criteria of ≥ 95% identity over an alignment of length ≥ 180 bp. If no such hit
190 was found the sequence was classified as “no match”. If multiple best hits were found and
191 these had conflicting taxonomies, the most detailed level of consensus taxonomy was
192 assigned to the OTU. After removal of pyrosequencing noise and chimeric sequences, 271
193 355 high quality, typically 198 bp long, sequence reads remained, with 1137-12909 reads per
194 sample (mean = 2261). These corresponded to 3597 unique sequences and 1818 OTUs,
195 clustered at 97% similarity level using complete linkage clustering. The majority (98%) of

196 reads was of clear bacterial origin and had an RDP relative within 95% sequence similarity.
197 Statistics on number of sequences and OTUs are presented in Table E1 (online repository).
198
199 **Statistical analysis**
200 Statistical significance testing over- and under-representation of the bacterial lineages was
201 made at phylum, class, genus, and OTU (3% dissimilarity) levels. Comparisons were made
202 using the Bioconductor *R* package (Empirical analysis of digital gene expression in R) EdgeR
203 [19], and p-values were converted to False Discovery Rate values (q-values) to correct for
204 multiple testing. [19] EdgeR is a statistical test that is designed for the analysis of replicated
205 count-based expression data. The Shannon diversity index was employed to measure the
206 biodiversity in samples. Briefly, it is a test that takes in account the number of species and the
207 evenness of the species, typically with a value between 1.5-3.5. [20] It was calculated as $-\Sigma$
208 $\log(p_i)p_i$, where p_i denotes the frequency of OTU $_i$ [21] and differences in this index were tested
209 with Mann-Whitney U-test in the R software (http://www.r-project.org/). Clustering of OTUs
210 was analyzed with Fast Unifrac (http://bmf2.colorado.edu/fastunifrac/) [22] by calculating
211 weighted sample distances.
212 Repeated-measures ANOVA was employed in analyses of multiple longitudinal measures of
213 a specific phylum or genus in subjects in two different groups The X^2 test was employed for
214 categorical data, unless the expected frequency for any cell was less than five, when Fisher´s
215 exact test was employed (SPSS 16.0, SPSS Inc, Chicago, IL, USA).
216

RESULTS

Infants who developed atopic eczema, *i.e.* IgE-associated eczema, had a lower diversity of the total microbiota and the bacterial phylum Bacteriodetes and its genus *Bacteroides* at one month of age than infants who did not have any allergic manifestation during the two first years of life (Table II). The diversity of the phylum Proteobacteria, comprising Gram negative bacteria, was also reduced in the atopic infants, significantly so at 12 months of age (Table II). Furthermore, these phyla and genera differed significantly between atopic and non-atopic infants with repeated-measures ANOVA including all sampling time points during the first year of life (one month, one week and 12 months: p=0.049 for the total microbiota, p=0.04 for Bacteroidetes, p= 0.02 for *Bacteroides* and p=0.02 for Proteobacteria). Probiotic supplementation was a potential confounder. Even after exclusion of the probiotic-treated infants, however, several significant differences and some statistical tendencies were still observed. (p=0.03 for the total microbiota, p=0.06 for proteobacteria, p=0.096 for Bacteroidetes, p= 0.03 for *Bacteroides* at 1 month, and p=0.06 for Proteobacteria and p=0.01 for Bacteroidetes and *Bacteroides* at 12 months, data not shown). Nine infants received antibiotics between two and twelve months. Excluding them did not affect the result at 12 months (p=0.02 for Proteobacteria).

The relative abundance of the dominant bacterial phyla at various ages is displayed in Figure 1. During the first month of life there was a high inter-subject variability (Figure E1, online repository) and no significant differences at the phylum level between infants who did and did not develop atopic eczema. The relative abundance of Bacteroidetes, Proteobacteria and Actinobacteria, the latter a phylum comprising bifidobacteria, which are associated with breastfeeding, was high in both groups. At 12 months, however, these phyla had declined and Firmicutes, comprising Gram positive aerobe and anaerobe bacteria, had become dominant

resembling an adult microbiota pattern. At this age the relative abundance of Proteobacteria was lower (Edge R test p=0.008, q=0.02) and Firmicutes tended to be higher (Edge R test p=0.06, q=0.10) in atopic than non-atopic infants (Table III). Infants that have received antibiotics or probiotics did not differ significantly in relative abundance from those that have not (data not shown). Despite this, the differences in relative abundance between healthy and atopic infants were more significant if infants receiving antibiotics were excluded (Edge R test p=0.01, q=0.02 for Firmicutes, p=0.005, q=0.02 for Proteobacteria and p=0.03, q=0.05 for Bacteroidetes at 12 months, data not shown). Excluding infants receiving probiotics did not affect the relative abundance significantly.

In order to compare our findings with previous reports, which often relate allergic disease with bacterial classes and genera rather than phyla, the relative abundance of the dominant bacterial classes and genera is presented in Table III. Since 144 genera were identified, p-values were converted to False Discovery Rate values (q-values) in order to correct for multiple testing. *Bifidobacterium, Bacteroides*, *Streptococcus, Enterococcus* and sequences collectively classified to unclassified *Enterobacteriaceae* were the most abundant genera, especially during the first month of life. There was no significant difference between atopics and non-atopics for any of the dominant bacterial genera, except for *Enterococcus* and *Peptostreptococcaceae Incertae Sedis*, which were more abundant in atopic infants at 12 months of age. Among less abundant genera (relative abundance <1%), only a few differed significantly between atopic and non-atopic infants after correcting for multiple testing. The microaerophilic Gram negative *Sutterella,* belonging to the phylum Proteobacteria, was more abundant in the non-atopic infants both at one and 12 months of age (healthy vs. atopic, mean % [SD]: 0.2 [0.4] vs. 0.006 [0.02], p=0.008, q=0.02 at one month; 0.3 [0.5] vs. 0.2 [0.5], p=0.006, q=0.02 at 12 months). The Gram negative anaerobe *Fusobacterium,* belonging to the

phylum Fusobacteria, was also more abundant in this group at 12 months of age (healthy vs. atopic, mean % [SD]: 0.01 [0.02] vs. 0.002 [0.009], p=0.006, q=0.02). On the other hand, the Gram positive anaerobes *Eggerthella*, belonging to Actinobacteria, and *Coprobacillus*, belonging to Firmicutes, were more abundant in the atopic infants at 12 months (healthy vs. atopic, mean % [SD]: 0.1 [0.2] vs. 0.8 [1.0], $p<0.001$, q=0.002, and 0.01 [0.04] vs. 0.4 [0.09], $p<0.001$, $q<0.001$, respectively). The Gram positive anaerobe *Peptoniphilus*, belonging to Firmicutes, was more abundant at one month of age in the atopic infants (healthy vs. atopic, mean % [SD]: 0 [0] vs. 0.002 [0.006], p=0.01, q=0.03).

DISCUSSION

Employing the new high-throughput 16S based molecular microbiology, we could confirm and extend previous findings, that low intestinal diversity during the first month of life is associated with an increased risk of subsequent atopic disease. [6 9 10] In contrast to previous studies, we could also show that the differences in diversity and relative abundance were attributed to specific bacterial phyla and genera, possibly because the sensitivity of our analyses was higher than in previous diversity studies. [6, 9, 10]At 12 months, the mean of OTUs/sample were 69 in our study, as compared to 8.5 bands/sample (in DGGE) in a recent the study by Bisgaard *et al.* [10] It is noteworthy that the most important differences appeared the first months of life, supporting the theory that factors influencing the early of maturation of the immune system might be especially important for subsequent allergy development. [23] The study, however, did not clarify the debate whether a low total diversity of the gut microbiota in early childhood is more important than altered prevalence of particular bacterial species in allergy development. Total diversity was important, but the differences in diversity and relative abundance seemed to be defined to specific bacteria.

The low diversity of the phylum Bacteroidetes and its genus *Bacteroides* in infants developing atopic eczema is consistent with previous studies, reporting low levels of these bacteria to be associated both with allergic disease [2] and factors associated with allergic disease, such as a Western lifestyle [11, 12] and caesarean section. [24] *Bacteroides* species have also been demonstrated to have anti-inflammatory properties. Thus, *Bacteroides fragilis* prevented the induction of colitis via suppression of the pro-inflammatory cytokines TNF and IL-23 in an experimental colitis model [25] and also mediated a conversion from CD4+ T cells into IL-10 producing Foxp3 T regulatory cells during commensal colonization eliciting mucosal tolerance in another mice model. [26] Furthermore, *Bacteroides thetaiotaomicron*

modulates the expression of a large quantity of genes involved in mucosal barrier reinforcement. [27, 28]

Although our results indicate that the microbial diversity is more important than the colonization with any particular bacteria, one bacterial phylum, Proteobacteria, appeared to be less abundant in the atopic infants. This phylum comprises Gram negative bacteria, typically with endotoxin (LPS) incorporated in their cell wall. Endotoxin elicits a Th1 response via the innate immune system by enhancing IL-12 production from monocytes and dendritic cells,[29] and low exposure to endotoxin has been associated with increased risk of atopic eczema. [30] Also, the low allergy prevalence among children growing up in farms and less affluent countries has been attributed to high endotoxin exposure. [31, 32] Thus, a strong endotoxin exposure may downregulate atopy-promoting Th2 responses, possibly causing the negative association between atopic eczema and high abundance and diversity of Proteobacteria in the present study.

Previously, bifidobacteria and clostridia, especially *Clostridium difficile,* have been associated with allergic disease. [3, 33] None of these bacteria were related to allergic disease in this study. Neither was *Clostridium* a dominant bacterial genus. However, there were other genera within the phylum Firmicutes that were more abundant in the atopic than the non-atopic infants. Interestingly, Firmicutes-have been associated to other conditions related to a westernized lifestyle, such as obesity. [12, 34]

Importantly, assessments of stool samples merely reflect luminal colonic microbiota and not necessarily the colonization of the small intestine, in which the major part of the gut immune system is situated. The higher oxygen content in the upper gut favors facultative bacteria such as streptococci and lactobacilli, [35] which therefore might be more important than our results indicate.

In conclusion, the results support the hypothesis that low microbial diversity early in life is associated with an increased risk for allergic disease. The importance of bacteria belonging to the phyla Bacteroidetes and Proteobacteria was corroborated, while the importance of other bacteria previously associated with allergic disease, such as bifidobacteria and clostridia, could not be confirmed.

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Tables

TABLE I. Descriptive data of children included in the study.

	Atopic eczema % (n)	Healthy % (n)	p-value*
Boys	60 (12)	50 (10)	0.53
First born	45 (9)	50 (10)	0.75
Caesarean delivery	15 (3)	0 (0)	0.23
Furred pets	0 (0)	5 (1)	1.00
Maternal atopy	85 (17)	90 (18)	1.00
Paternal atopy	70 (14)	60 (12)	0.51
Breastfeeding			
1 month	100 (20)	100 (20)	1.00
12 months	25 (5)	35 (7)	0.49
Antibiotics			
1-12 months	15 (3)	30 (6)	0.45
12-24 months	50 (10)	30 (6)	0.20
Day-care			
0-12 months	0 (0)	5 (1)	1.00
12-24 months	70 (14)	85 (17)	0.45
Probiotic group	30 (6)	55 (11)	0.11

X^2 test. Fisher's exact test was used when the expected frequency for any cell was less than five

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TABLE II. The Shannon diversity index of the total microbiota, dominant phyla and significant genera in stool samples obtained at various ages from infants who did or did not develop atopic eczema during the first two years of life.

	Atopic eczema		Healthy		P-value*
	n=20 median	iqr**	n=20 median	iqr**	
1 week					
Total microbiota	1.59	1.33-1.77	1.58	1.42-1.83	0.78
Firmicutes	0.81	0.48-1.27	0.86	0.51-1.10	0.53
Proteobacteria	0.15	0.03-0.30	0.32	0.05-0.37	0.19
Actinobacteria	0.29	0.07-0.41	0.27	0.10-0.37	0.58
Bacteroidetes	0.02	0.00-0.51	0.20	0.00-0.39	0.60
1 month					
Total microbiota	1.47	1.16-1.66	1.69	1.53-2.15	0.004
Firmicutes	0.55	0.34-1.11	0.61	0.44-0.92	0.72
Proteobacteria	0.15	0.06-0.35	0.27	0.12-0.33	0.29
Actinobacteria	0.36	0.12-0.46	0.42	0.20-0.67	0.26
Bacteroidetes	0.05	0.00-0.36	0.48	0.08-0.60	0.02
Bacteroides	0.01	0.00-0.28	0.44	0.08-0.49	0.01
12 months					
Total microbiota	2.90	2.25-3.30	2.62	2.22-3.27	0.65
Firmicutes	2.31	1.71-2.58	1.89	1.49-2.39	0.12
Proteobacteria	0.04	0.01-0.07	0.07	0.04-0.13	0.02
Actinobacteria	0.21	0.11-0.41	0.17	0.02-0.38	0.43
Bacteroidetes	0.16	0.03-0.36	0.50	0.12-0.65	0.08

*Mann Whitney U-test. ** interquartile range

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TABLE III. The mean of the relative abundance of dominant phyla (bold), classes and genera (relative abundance >1% at any age) in stool samples obtained at various ages from infants who did or did not develop atopic eczema (AE) during the first two years of life.

	1 week		1 month		12 months	
	Healthy	**AE**	**Healthy**	**AE**	**Healthy**	**AE**
	n=20 mean % (SD)	n=20 mean % (SD)	n=20 mean % (SD)	n=20 mean % (SD)	n=20 mean % (SD)	n=20 mean % (SD)
Actinobacteria	21 (23)	28 (27)	31 (22)	43 (35)	14 (20)	11 (12)
Bifidobacterium	21 (23)	28 (27)	29 (22)	41 (35)	14 (20)	10 (11)
Collinsella	<1	<1	1 (3)	<1	<1	<1
Proteobacteria	20 (20)	14 (18)	12 (10)	12 (15)	# 4 (7)	# 1 (2)
Gammaproteobacteria	20 (21)	13 (24)	12 (29)	12 (22)	3 (5)	1 (3)
Enterobacteriaceae (unclassified)	18 (21)	8 (15)	7 (11)	5 (10)	2 (4)	<1
Bacteriodetes	15 (21)	12 (18)	24 (22)	9 (15)	15 (12)	7 (9)
Bacteroides	14 (21)	11 (16)	21 (22)	7 (13)	13 (12)	6 (6)
Parabacteroides	1 (3)	2 (4)	2 (4)	<1	<1	<1
Prevotella	<1	<1	<1	<1	<1	1 (5)
Firmicutes	43 (28)	45 (33)	32 (22)	35 (32)	65 (19)	74 (16)
Bacilli	25 (25)	29 (31)	14 (20)	16 (26)	5 (20)	6 (27)
Streptococcus	12 (10)	10 (17)	9 (9)	11 (14)	5 (9)	2 (5)
Enterococcus	5 (11)	9 (16)	1 (3)	3 (6)	**<1	**4 (14)
Lactobacillus	<1	2 (4)	2 (4)	<1	<1	<1
Clostridia	18 (25)	15 (24)	16 (26)	18 (30)	55 (20)	65 (18)
Veillonella	3 (8)	2 (4)	2 (2)	3 (6)	2 (3)	1 (2)
Lachnospiraceae Incertae Sedis	1 (3)	<1	<1	1 (6)	4 (5)	7 (6)
Peptostreptococcaceae Incertae Sedis	1 (2)	1 (4)	<1	<1	*3 (3)	*5 (4)
Erysipelotrichaceae Incertae Sedis	<1	<1	<1	2(6)	4 (4)	4 (6)
Clostridium	<1	<1	2 (8)	2 (6)	1 (4)	<1
Lachnospiraceae	<1	<1	<1	<1	7 (7)	6 (6)
Faecalibacterium	<1	<1	<1	<1	3 (4)	2 (4)
Ruminococcus	<1	<1	<1	<1	1 (2)	3 (3)
Anaerostipes	<1	<1	<1	<1	1 (4)	1 (3)
Erysipelotrichi	<1	<1	3 (19)	2 (26)	4 (7)	9 (3)
Verrucomicrobia	<0.1	<0.1	1 (2)	<0.1	2 (4)	1 (4)
Akkermansia	<1	<1	1 (5)	<1	2 (4)	2 (4)

Edge p-value=0.01, q-value=0.03, *edge p-value=0.02,q-value=0.04, **edge p-value=0.002, q-value=0.005.

478 **Legends to figures.**

479

480 **FIG 1.**

481 Relative abundance of dominant bacterial phyla in stool samples in each subject at one week

482 (a) and at one (b) and 12 months (c) of age in 20 infants who developed atopic eczema and 20

483 infants without any allergic manifestations.

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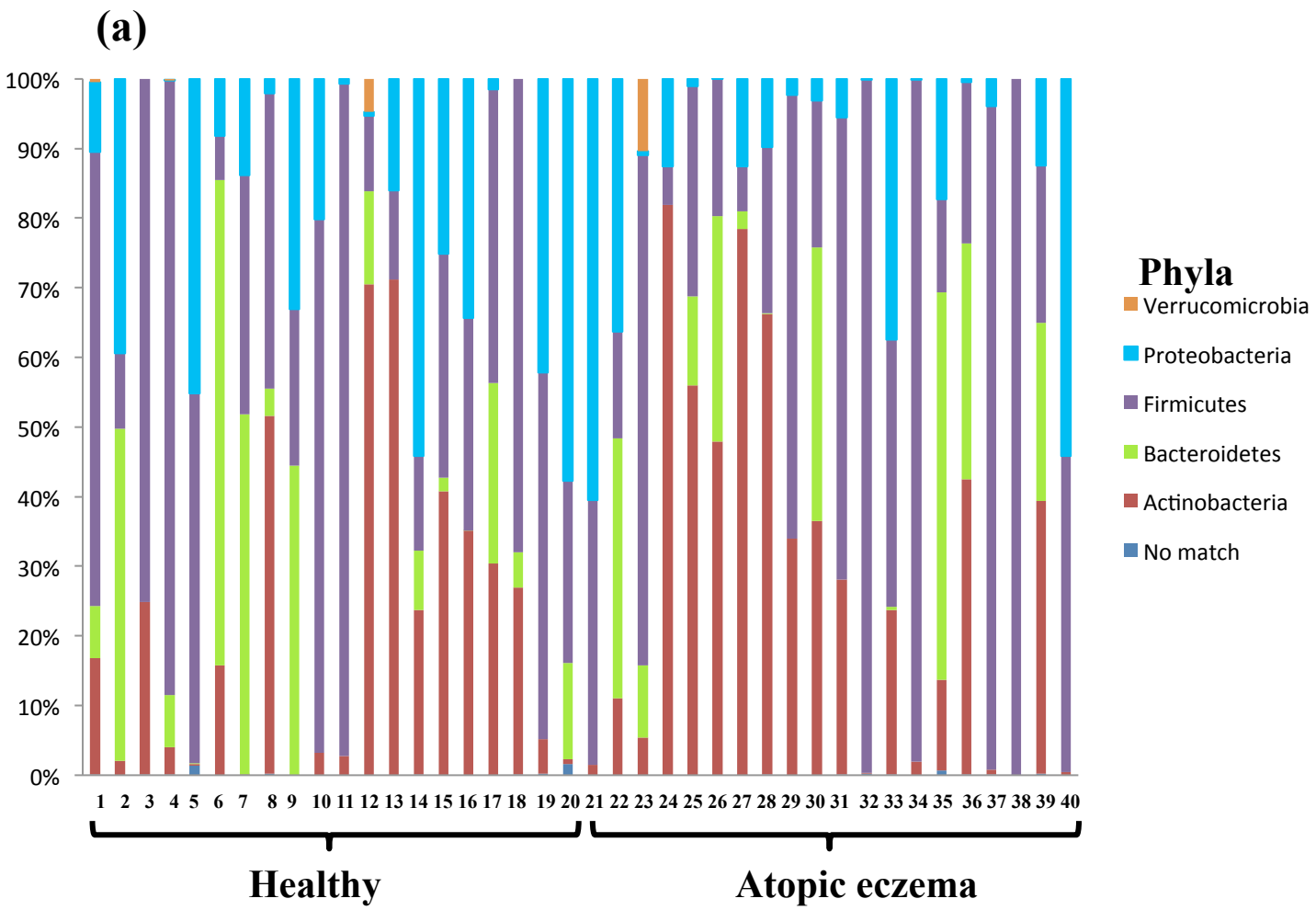
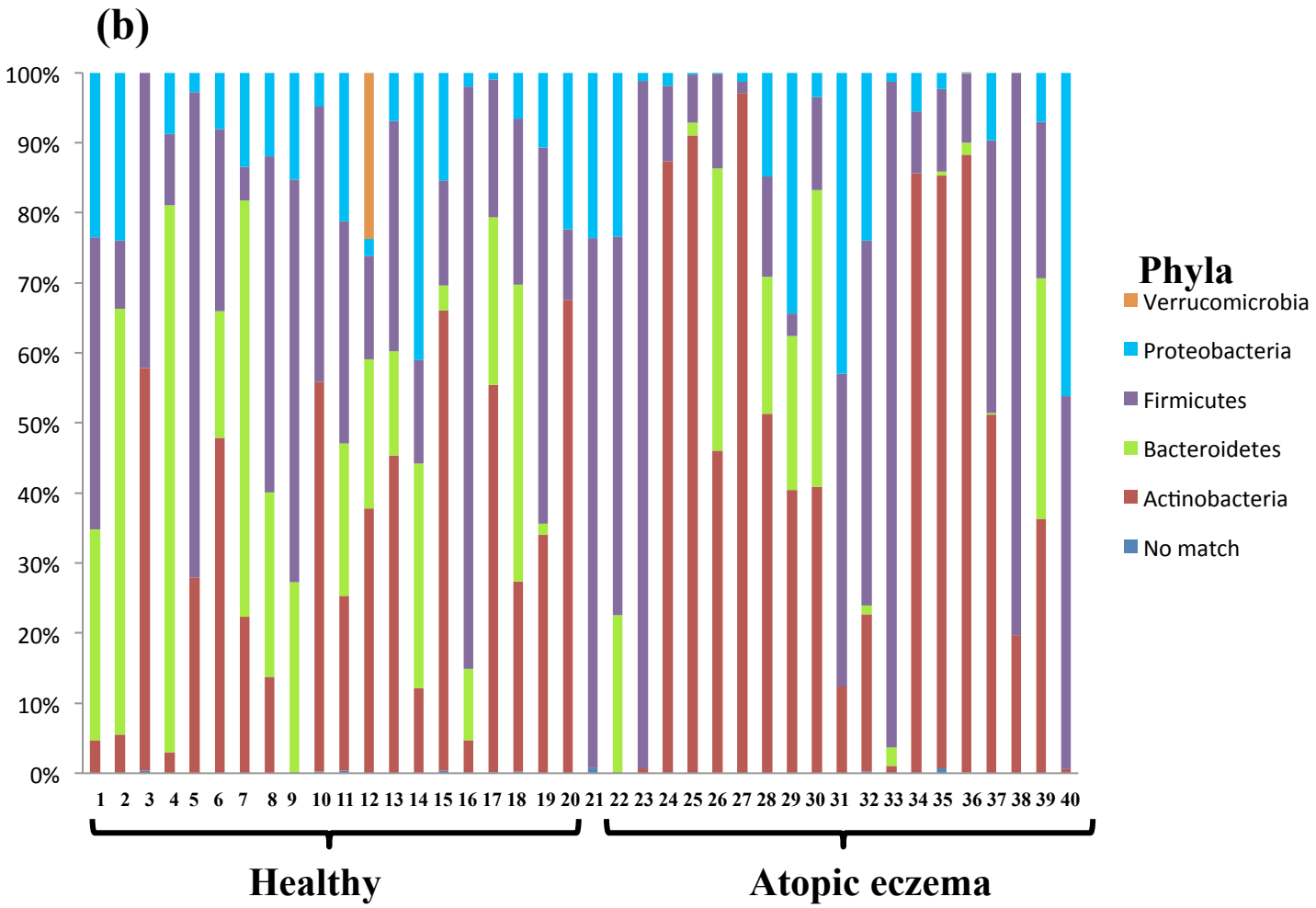

(a)
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40
Healthy
Atopic eczema
Phyla
Verrucomicrobia
Proteobacteria
Firmicutes
Bacteroidetes
Actinobacteria
No match


(b)
100%
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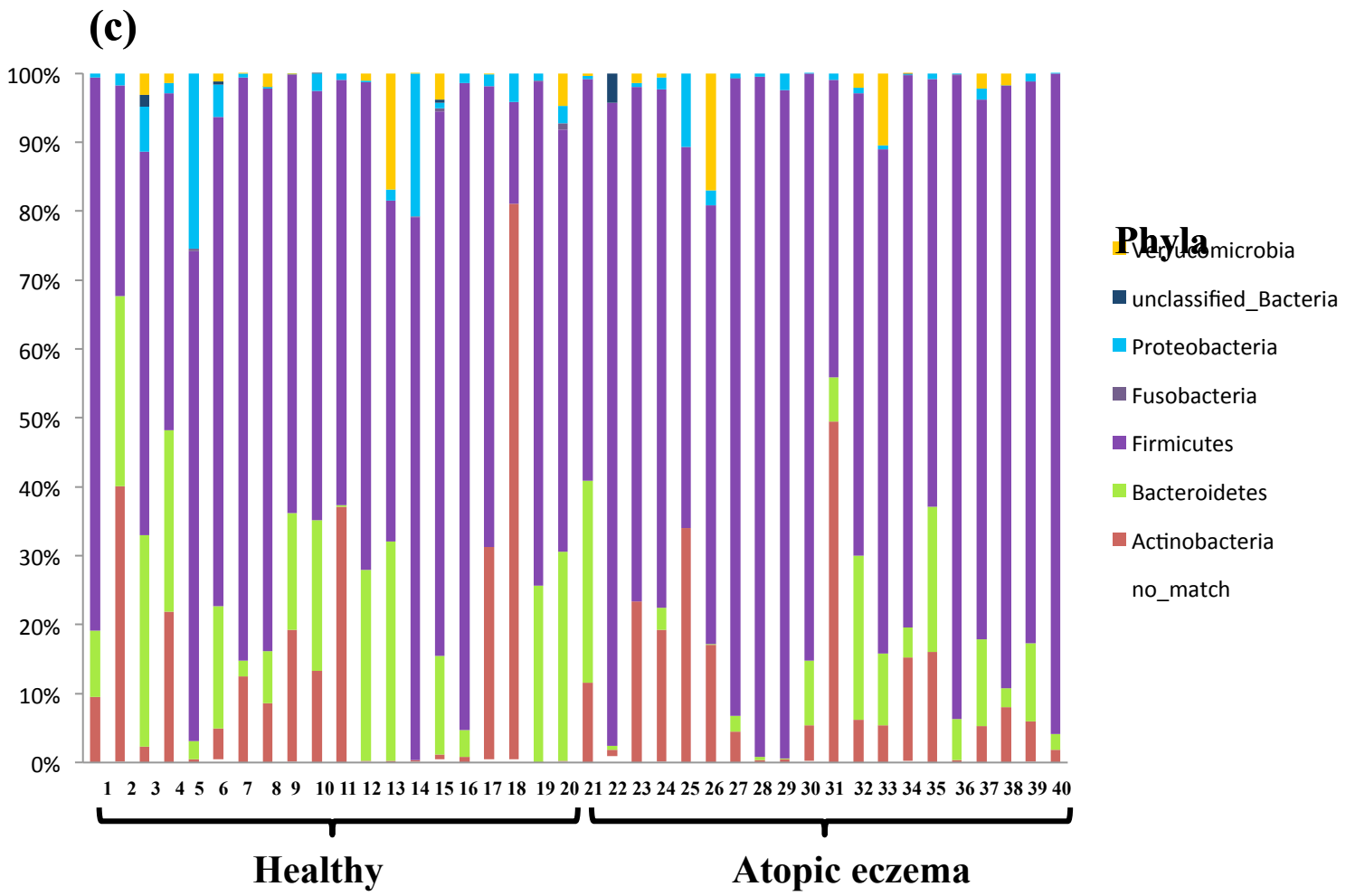
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40
Healthy
Atopic eczema
Phyla
Verrucomicrobia
Proteobacteria
Firmicutes
Bacteroidetes
Actinobacteria
No match


(c)
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40
Healthy
Atopic eczema
Phyla
Verrucomicrobia
unclassified_Bacteria
Proteobacteria
Fusobacteria
Firmicutes
Bacteroidetes
Actinobacteria
no_match