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Linköping University Post Print



N.B.: When citing this work, cite the original article.

Original Publication:

Thomas Abrahamsson, Hedvig E Jakobsson, Anders F Andersson, Bengt Bjorksten, Lars Engstrand and Maria Jenmalm, Low diversity of the gut microbiota in infants with atopic eczema, 2012, Journal of Allergy and Clinical Immunology, (129), 2, 434-440.
<http://dx.doi.org/10.1016/j.jaci.2011.10.025>

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Postprint available at: Linköping University Electronic Press

<http://urn.kb.se/resolve?urn=urn:nbn:se:liu:diva-75901>

1 **Low diversity of the gut microbiota in infants developing atopic eczema**

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33 Supported by grants from BioGaia AB, Stockholm, Sweden, the Ekhaga Foundation, the
34 Heart and Lung foundation, the Research Council for the South-East Sweden (grant No.
35 F2000-106), The Olle Engqvist Foundation, the Swedish Asthma and Allergy Association,
36 the Swedish Research Council, the University Hospital of Linköping, the Söderberg
37 Foundation, the Vårdal Foundation for Health Care Science and Allergy Research, Sweden.

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39 Total word count: 2956

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41

42 **ABSTRACT**

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

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

50 **Methods:** The microbial diversity and composition was analyzed with barcoded 16S rDNA
51 454-pyrosequencing in stool samples at one week, one month and 12 months of age in 20
52 infants developing IgE-associated eczema and 20 infants without any allergic manifestation
53 until two years of age. (ClinicalTrials.gov ID NCT01285830)

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

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

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

65

66 **Capsule summary**

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

70

71 **Key words**

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

74

75 **Abbreviations**

76 BLAST: Basic Local Alignment Search Tool

77 CV: Coefficient of variance

78 DGGE: Denaturing gradient gel electrophoresis

79 Edge R: Empirical analysis of digital gene expression in R

80 FISH: Fluorescent in situ hybridization

81 LPS: Lipopolysaccharides

82 OTU: Operational Taxonomic Unit

83 RDP: Ribosomal Database Project

84 SPT: skin prick test

85 T-RFLP: Terminal restriction fragment length polymorphism

86

87 **INTRODUCTION**

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

97

98 As an alternative explanation, it has been suggested that low diversity of intestinal microbiota
99 would explain the increase of allergic disease in affluent societies.^{6,7} The underlying
100 rationale is that the gut immune system reacts to exposure to new bacterial antigens and
101 repeated exposure would enhance the development of immune regulation. Although this
102 theory emerged more than a decade ago,⁸ there are still only few studies relating the diversity
103 with allergy, likely due to methodology limitations. In three studies employing molecular
104 techniques, terminal restriction fragment length polymorphism (T-RFLP)⁶ and denaturing
105 gradient gel electrophoresis (DGGE)^{9,10}, infants developing sensitization¹⁰ or eczema^{6,9}
106 were reported to have fewer peaks/bands than healthy ones. Yet, no specific microbes were
107 identified with these molecular methods. Furthermore, the sensitivity of the methods appears
108 to be low, since the median number of peaks/bands was much lower than the expected
109 number of bacterial species.^{6,9,10}

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

112 Previously uncultivated bacteria can now be detected, and there is no need to decide what
113 bacteria to analyze in advance. Thus the assessment can be made unprejudiced. This will
114 allow more comprehensive knowledge of the intestinal microbiota and its impacts on the
115 immune system. We have employed barcoded 16S rRNA 454-pyrosequencing¹³ to assess the
116 microbial diversity and characterize the dominant bacteria in stool during the first year of life
117 in infants who either developed atopic eczema or did not have any allergic manifestation up to
118 two years of age.

119

120

121

122 **METHODS**

123 **Study design**

124 The infants included in this study were part of a larger study in South Eastern Sweden
125 between 2001 and 2005, evaluating allergy prevention with the probiotic *Lactobacillus*
126 *reuteri* ATCC 55730.¹⁴ In this study the infant received probiotics or placebo daily from day
127 1-3 until 12 months of age. Clinical follow-ups were done at 1, 3, 6, 12 and 24 months of age
128 and telephone interviews at 2, 4, 5, 8, 10 and 18 months. A questionnaire was completed on
129 each occasion. Stool samples were collected from the infants at age 5-7 days and at one
130 month and 12 months of age. The samples were immediately frozen at -20°C following
131 collection and later stored at -70°C. Among the 188 infants completing the original study and
132 from which stool samples were available from all three sampling occasions, 20 infants with
133 atopic eczema and 20 without any allergic manifestation were randomly selected to this study.

134

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

143

144 **Diagnostic criteria of atopic eczema**

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

147 with eczema also was sensitized.¹⁵ Infants were regarded as sensitized if they had at least one
148 positive SPT and/or detectable circulating allergen specific IgE antibodies. Skin prick tests
149 were done on the volar aspects of the forearm with egg white, fresh skimmed cow milk (lipid
150 concentration 0.5%) and standardised cat, birch and timothy extracts (Soluprick®, ALK,
151 Hørsholm, Denmark) at 6, 12 and 24 months of age. Histamine hydrochloride (10 mg/ml) was
152 used as positive and albumin diluents as negative control. The test was regarded as positive if
153 the mean diameter of the wheal was >3mm. Circulating IgE antibodies to egg white and
154 cow's milk were analysed at 6, 12, and 24 months of age in venous blood (UniCap®
155 Pharmacia CAP System™, Pharmacia Diagnostics, Uppsala, Sweden). The cut off level was
156 0.35 kU/L, according to the protocol of the manufacturer. In addition, circulating IgE to a
157 mixture of food allergens, including egg white, cow's milk, cod, wheat, peanut and soy bean,
158 was analysed at 6, 12 and 24 months of age (UniCap® Pharmacia CAP System™, fx5,
159 Pharmacia Diagnostics).

160

161 **DNA extraction, 16S rRNA gene amplification, and sequencing**

162 Extraction of bacterial DNA from the fecal samples and the 16S rRNA gene amplification
163 was made according to a previous publication¹³ with the following modifications; the primer
164 pair used, targeting the variable regions 3 and 4 of the 16S rRNA gene, were 341f
165 5'CCTACGGGNGGCWGCAG with adaptor B and 805r
166 5'GACTACHVGGGTATCTAATCC with adaptor A¹⁶ and sample-specific sequence
167 barcodes consisting of five nucleotides. The barcodes contained no homopolymers and a pair
168 of barcodes differed in at least two positions. A negative PCR reaction without template was
169 also included for all primer pairs in each run. The PCR was run for 25 cycles. The PCR-
170 products with proximal lengths of 450 bp were purified with AMPure beads (Becton
171 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 package¹⁷ 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 $\geq 95\%$ 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 $-\sum$

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

217
218**RESULTS**

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

235

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

243 resembling an adult microbiota pattern. At this age the relative abundance of Proteobacteria
244 was lower (Edge R test $p=0.008$, $q=0.02$) and Firmicutes tended to be higher (Edge R test
245 $p=0.06$, $q=0.10$) in atopic than non-atopic infants (Table III). Infants that have received
246 antibiotics or probiotics did not differ significantly in relative abundance from those that have
247 not (data not shown). Despite this, the differences in relative abundance between healthy and
248 atopic infants were more significant if infants receiving antibiotics were excluded (Edge R
249 test $p=0.01$, $q=0.02$ for Firmicutes, $p=0.005$, $q=0.02$ for Proteobacteria and $p=0.03$, $q=0.05$
250 for Bacteroidetes at 12 months, data not shown). Excluding infants receiving probiotics did
251 not affect the relative abundance significantly.

252

253 In order to compare our findings with previous reports, which often relate allergic disease
254 with bacterial classes and genera rather than phyla, the relative abundance of the dominant
255 bacterial classes and genera is presented in Table III. Since 144 genera were identified, p -
256 values were converted to False Discovery Rate values (q -values) in order to correct for
257 multiple testing. *Bifidobacterium*, *Bacteroides*, *Streptococcus*, *Enterococcus* and sequences
258 collectively classified to unclassified *Enterobacteriaceae* were the most abundant genera,
259 especially during the first month of life. There was no significant difference between atopics
260 and non-atopics for any of the dominant bacterial genera, except for *Enterococcus* and
261 *Peptostreptococcaceae Incertae Sedis*, which were more abundant in atopic infants at 12
262 months of age. Among less abundant genera (relative abundance $<1\%$), only a few differed
263 significantly between atopic and non-atopic infants after correcting for multiple testing. The
264 microaerophilic Gram negative *Sutterella*, belonging to the phylum Proteobacteria, was more
265 abundant in the non-atopic infants both at one and 12 months of age (healthy vs. atopic, mean
266 % [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],
267 $p=0.006$, $q=0.02$ at 12 months). The Gram negative anaerobe *Fusobacterium*, belonging to the

268 phylum Fusobacteria, was also more abundant in this group at 12 months of age (healthy vs.
269 atopic, mean % [SD]: 0.01 [0.02] vs. 0.002 [0.009], $p=0.006$, $q=0.02$). On the other hand, the
270 Gram positive anaerobes *Eggerthella*, belonging to Actinobacteria, and *Coprobacillus*,
271 belonging to Firmicutes, were more abundant in the atopic infants at 12 months (healthy vs.
272 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],
273 $p<0.001$, $q<0.001$, respectively). The Gram positive anaerobe *Peptoniphilus*, belonging to
274 Firmicutes, was more abundant at one month of age in the atopic infants (healthy vs. atopic,
275 mean % [SD]: 0 [0] vs. 0.002 [0.006], $p=0.01$, $q=0.03$).

276

277

278 **DISCUSSION**

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

293

294 The low diversity of the phylum Bacteroidetes and its genus *Bacteroides* in infants
295 developing atopic eczema is consistent with previous studies, reporting low levels of these
296 bacteria to be associated both with allergic disease² and factors associated with allergic
297 disease, such as a Western lifestyle^{11,12} and caesarean section.²⁴ *Bacteroides* species have
298 also been demonstrated to have anti-inflammatory properties. Thus, *Bacteroides fragilis*
299 prevented the induction of colitis via suppression of the pro-inflammatory cytokines TNF and
300 IL-23 in an experimental colitis model²⁵ and also mediated a conversion from CD4+ T cells
301 into IL-10 producing Foxp3 T regulatory cells during commensal colonization eliciting
302 mucosal tolerance in another mice model.²⁶ Furthermore, *Bacteroides thetaiotaomicron*

303 modulates the expression of a large quantity of genes involved in mucosal barrier
304 reinforcement.^{27, 28}

305

306 Although our results indicate that the microbial diversity is more important than the
307 colonization with any particular bacteria, one bacterial phylum, Proteobacteria, appeared to be
308 less abundant in the atopic infants. This phylum comprises Gram negative bacteria, typically
309 with endotoxin (LPS) incorporated in their cell wall. Endotoxin elicits a Th1 response via the
310 innate immune system by enhancing IL-12 production from monocytes and dendritic cells,²⁹
311 and low exposure to endotoxin has been associated with increased risk of atopic eczema.³⁰

312 Also, the low allergy prevalence among children growing up in farms and less affluent
313 countries has been attributed to high endotoxin exposure.^{31, 32} Thus, a strong endotoxin
314 exposure may downregulate atopy-promoting Th2 responses, possibly causing the negative
315 association between atopic eczema and high abundance and diversity of Proteobacteria in the
316 present study.

317

318 Previously, bifidobacteria and clostridia, especially *Clostridium difficile*, have been associated
319 with allergic disease.^{3, 33} None of these bacteria were related to allergic disease in this study.

320 Neither was *Clostridium* a dominant bacterial genus. However, there were other genera within
321 the phylum Firmicutes that were more abundant in the atopic than the non-atopic infants.

322 Interestingly, Firmicutes have been associated to other conditions related to a westernized
323 lifestyle, such as obesity.^{12, 34}

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

329

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

335

336 **Acknowledgements**

337 We thank Mrs Lena Lindell, Mrs Elisabeth Andersson, Mrs Linnea Andersson and Mrs Eivor
338 Folkesson, Dr Göran Oldaeus and Dr Ted Jacobsson for their brilliant and enthusiastic work
339 guiding the families through the study and all the sampling procedures. We also thank Mrs
340 Anne-Marie Fornander for excellent technical assistance and Christopher Quince for assisting
341 with sequence noise removal.

342

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443 **Tables**444 **TABLE I.** Descriptive data of children included in the study.

	Atopic eczema % (n)	Healthy % (n)	p-value*
449 Boys	60 (12)	50 (10)	0.53
450 First born	45 (9)	50 (10)	0.75
451 Caesarean delivery	15 (3)	0 (0)	0.23
452 Furred pets	0 (0)	5 (1)	1.00
453 Maternal atopy	85 (17)	90 (18)	1.00
454 Paternal atopy	70 (14)	60 (12)	0.51
455			
456 Breastfeeding			
457 1 month	100 (20)	100 (20)	1.00
458 12 months	25 (5)	35 (7)	0.49
459			
460 Antibiotics			
461 1-12 months	15 (3)	30 (6)	0.45
462 12-24 months	50 (10)	30 (6)	0.20
463			
464 Day-care			
465 0-12 months	0 (0)	5 (1)	1.00
466 12-24 months	70 (14)	85 (17)	0.45
467			
468 Probiotic group	30 (6)	55 (11)	0.11

469
470 X^2 test. Fisher's exact test was used when the expected
471 frequency for any cell was less than five

472
473

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
<i>Bacteroides</i>	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

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)
<i>Bifidobacterium</i>	21 (23)	28 (27)	29 (22)	41 (35)	14 (20)	10 (11)
<i>Collinsella</i>	<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)
<i>Enterobacteriaceae</i>						
(unclassified)	18 (21)	8 (15)	7 (11)	5 (10)	2 (4)	<1
Bacterioidetes	15 (21)	12 (18)	24 (22)	9 (15)	15 (12)	7 (9)
<i>Bacteroides</i>	14 (21)	11 (16)	21 (22)	7 (13)	13 (12)	6 (6)
<i>Parabacteroides</i>	1 (3)	2 (4)	2 (4)	<1	<1	<1
<i>Prevotella</i>	<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)
<i>Streptococcus</i>	12 (10)	10 (17)	9 (9)	11 (14)	5 (9)	2 (5)
<i>Enterococcus</i>	5 (11)	9 (16)	1 (3)	3 (6)	**<1	**4 (14)
<i>Lactobacillus</i>	<1	2 (4)	2 (4)	<1	<1	<1
Clostridia	18 (25)	15 (24)	16 (26)	18 (30)	55 (20)	65 (18)
<i>Veillonella</i>	3 (8)	2 (4)	2 (2)	3 (6)	2 (3)	1 (2)
<i>Lachnospiraceae</i>						
<i>Incertae Sedis</i>	1 (3)	<1	<1	1 (6)	4 (5)	7 (6)
<i>Peptostreptococcaceae</i>						
<i>Incertae Sedis</i>	1 (2)	1 (4)	<1	<1	*3 (3)	*5 (4)
<i>Erysipelotrichaceae</i>						
<i>Incertae Sedis</i>	<1	<1	<1	2(6)	4 (4)	4 (6)
<i>Clostridium</i>	<1	<1	2 (8)	2 (6)	1 (4)	<1
<i>Lachnospiraceae</i>	<1	<1	<1	<1	7 (7)	6 (6)
<i>Faecalibacterium</i>	<1	<1	<1	<1	3 (4)	2 (4)
<i>Ruminococcus</i>	<1	<1	<1	<1	1 (2)	3 (3)
<i>Anaerostipes</i>	<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)
<i>Akkermansia</i>	<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.**

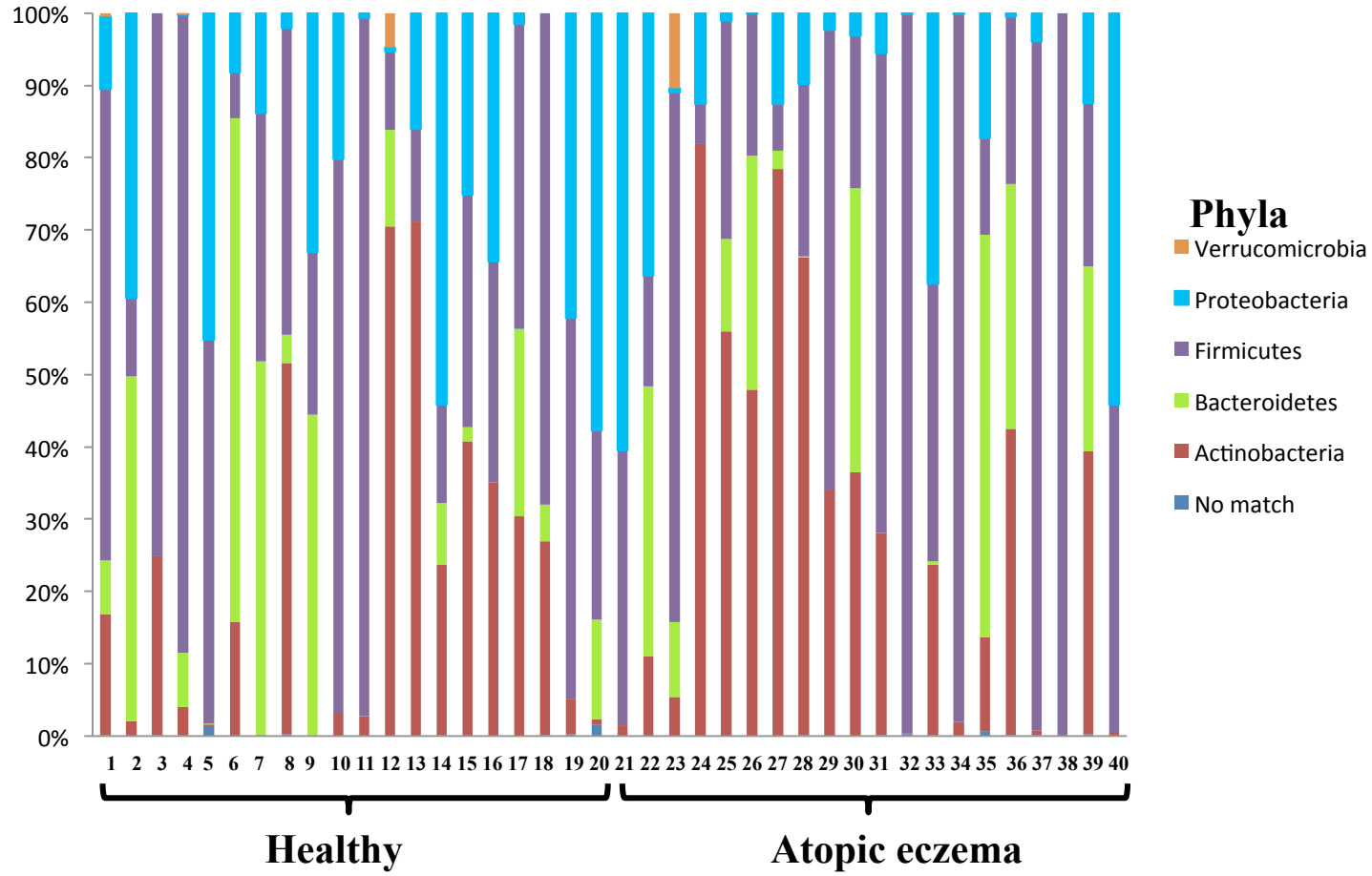
481 Relative abundance of dominant bacterial phyla in stool samples in each subject at one week

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

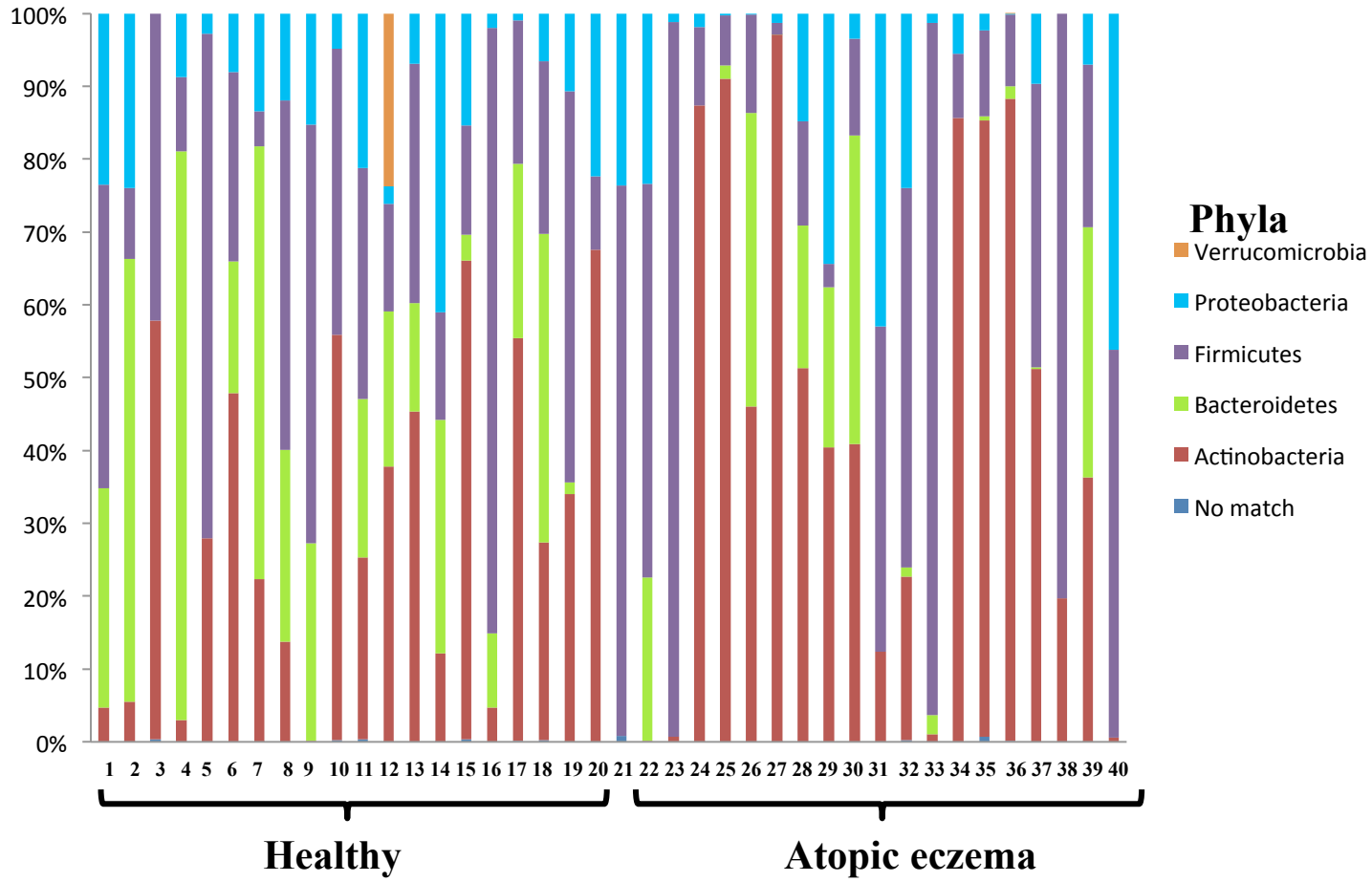
483 infants without any allergic manifestations.

484

(a)



(b)



(c)

