Human seroreactivity to gut microbiota antigens

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TITLE: Human seroreactivity to gut microbiota antigens

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ABSTRACT:

Background: While immune responses directed against antigens from the intestinal microbiota are observed in certain diseases, the normal human adaptive immune response to intestinal microbiota is poorly defined.

Objective: Our goal was to assess the adaptive immune response to the intestinal microbiota present in 143healthy adults andcompare this response to the immune response observed in 52 children and their mothers at risk of having allergic disease.

Methods: Human serum was collected from adults and from children followed from birth to seven years of age, and the serum IgG response to a panel of intestinal microbiota antigens was assessed using a novel protein microarray.

Results: Nearly every individual tested, regardless of health status, had serum IgG that recognized a common set of antigens. Seroreactivity to the panel of antigens was significantly lower in atopic adults. Healthy infants expressed the highest level of IgG seroreactivity to intestinal microbiota antigens. This adaptive response developed between 6 and 12 months of age, and peaked around 2 years of age. Low IgG responses to certain clusters of microbiota antigens during infancy were associated with allergy development during childhood.

Conclusions: There is an observed perturbation of the adaptive response to antigens from the microbiota in allergic individuals. These perturbations are observable even in childhood, suggesting that optimal stimulation of the adaptive immune system by the microbiota may be needed to prevent certain immune-mediated diseases.

KEY MESSAGES:

[1] We describe the development of adaptive immune responses against antigens from the intestinal microbiota, and we show that immune activation against antigens from the gut microbiota is normal and present from infancy into adulthood.

[2] In allergic individuals, we show that there is a significant decrease in seroreactivity to particular groups of microbiota antigens, and low seroreactivity during infancy associates with allergy development during childhood.

CAPSULE SUMMARY:

In healthy individuals, there is a IgG seroreactivity to antigens from the gut microbiota, and this response appears to be decreased in allergy.

KEY WORDS: Adaptive; atopy; allergy; childhood; IgG; microarray; microbiota; Antigens, Bacterial; Antibodies, Bacterial

INTRODUCTION

The intestinal microbiota has become a major focal point in the study of many immunologic diseases, and advances in the characterization of the gut microbiota have identified patterns of colonization associated with disease severity and pathogenesis. Multiple autoimmune and inflammatory diseases have been linked to alterations in the gut microbiota (1, 2). The symbiotic relationship between the microbiota and the human host begins at birth (3). The microbiota rapidly expands and changes before converging to a stable colonization pattern (4-6). The developing microbiota informs the immune system by modulating inflammatory gene expression (7), and microbial colonization is necessary for the development of normal immune structures (8, 9). Even in the mature immune system, the microbiota exerts a powerful influence by maintaining immune homeostasis through the regulation of various lineages of T cells (10-14). Despite the overall stability of gut microbiota colonization in individuals (15), the species composition appears to vary among individuals (16). This variation may be beneficial, because a less diverse gut microbiota is present during the first month of life in infants later developing atopic eczema (17) and asthma (18). The diversity of the microbiota in healthy individuals, coupled with the known influence of the microbiota on immune homeostasis, suggests that the specific makeup of the microbiota may be of less importance than the body's adaptive immune response to the microbiota itself.

Much effort has been expended to characterize the microbiota in healthy adults (19, 20), including the evolution of microbial colonization in a healthy infant from birth to 3 years of age (21), but the development of the normal human adaptive immune response to the human microbiota is less understood. To this end, we developed a novel protein microarray to investigate the interplay between the adaptive immune system

88 and the gut microbiota and categorized the IgG seroreactivity of individuals from the
89 United States, Canada, and Sweden to a panel of antigens from the gut microbiota.
90
91 **MATERIALS AND METHODS**
92 **Serum samples.** Serum samples were collected with parental consent from 52
93 Swedish children and their mothers one week post-partum, as well as 70 healthy adults
94 in Linköping, Sweden, 43 in Birmingham, AL, USA and 30 in Winnipeg, MB, Canada.
95 The mothers and children participated in an allergy prevention study, where
96 *Lactobacillus reuteri* (ATCC 55730; 1 x 10^8 CFU/day, BioGaia AB, Stockholm, Sweden)
97 or placebo was administered to the mother from gestational week 36 and to the infant
98 through the first year of life (22). At least one family member of the child had an allergic
99 disease. The background factors and allergic manifestations in these children until seven
100 years of age are described in Table 1. Non-atopic controls participated in an
101 investigation of immune responses to paternal antigens during pregnancy (23). For
102 Swedish mothers, the median age was 29 years (range 21 to 44 years). For Birmingham
103 adults, the median age was 32 (range 20 to 76; 56% males/ 44% female). Samples were
104 obtained with consent. For Winnipeg adults, the median age was 43 (range 17 to 75;
105 41% male, 59% female). Sera collected from patients with Crohn's disease in
106 Birmingham (N=10) and Winnipeg (N=30) were used in some experiments for
107 comparison with healthy and allergic sera for reactivity to flagellin antigens.
108 **Microbiota antigen microarray.** Proteins were diluted in TRIS buffer pH 8.0
109 with 0.5% SDS at 0.2 mg/ml. The proteins were printed onto FAST 16 nitrocellulose
110 pad slides (Whatman) using a MicroGrid II robot (Genomic Solutions) in duplicate in
111 two different parts of the pad. Thus each antigen is present in quadruplicate. The

112 printed slides were allowed to air-dry over night. Slides were blocked (Protein Array
113 Blocking Buffer – Whatman), probed with human sera at 1:100 dilution, washed, and
114 incubated with Alexa 647- or Alexa 546-labeled goat anti-human IgG or IgA (KPL) . The
115 proteins included in the microarray are listed in Table 1.

116 **Analysis of microarray data.** Software programs that were developed for
117 analysis of DNA microarrays were used to analyze the data from the microbiota antigen
118 array. The slides are read in an Axon GenePix 4000B dual laser microarray reader. The
119 accompanying GenePix Pro 6.0 software determines the net median pixel intensities for
120 each individual feature (antigen spots) from a set of 10 measurements/feature. The
121 instrument and software automatically subtracted the pixel intensities of the
122 background area surrounding the feature. A median net digital fluorescence unit (DFU)
123 for each feature represents the median values from 4 replicate antigen features on each
124 array. Statistical analysis of data was performed with R statistical package or GraphPad
125 Prism using appropriate tests to compare values between groups. Analysis of the data
126 was done without and with a Bonferoni correction for multiple comparisons; the p-
127 values were highly significant with both approaches. The p-values in the text and
128 figures are the analyses uncorrected for multiple comparisons.

129 Sequences from the antigens obtained from murine cecum were compared to
130 human microbiota sequences present in the following databases: NIH Human
131 Microbiome Project (http://www.hmpdacc.org), NCBI Gene Bank
132 (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and the metagenome gene catalog (Reference
133 20).

134 **Clinical features and definitions of allergic children.** Allergic
135 manifestations included eczema, recurrent wheeze, allergic rhinoconjunctitivis (ARC),

allergic urticaria, gastrointestinal allergy and IgE sensitization against food or other allergens. A diagnosis of eczema was defined as a pruritic, chronic or chronically relapsing non-infectious dermatitis with typical features and distribution. An asthma diagnosis required at least one of following two criteria: 1. Doctor diagnosis and asthma symptoms and/or medication during the last twelve months; 2. Wheeze or nocturnal cough and a positive reversibility test and/or pathological FENO value. In Sweden most children with asthma are asymptomatic when visiting the doctor, since they are efficiently treated with inhaled corticosteroids. If the asthma diagnosis was based on doctors diagnosis, medical records of the child was always reviewed to confirm that the diagnosis were consistent with the GINA criteria (http://www.ginasthma.com). The diagnosis of ARC was based on standard ISAAC question (http://isaac.auckland.ac.nz/Index.html) and required watery discharge at least twice in contact with the same allergen and no signs of infection. The diagnosis of gastrointestinal allergy required vomiting, diarrhea, or systemic reaction after ingestion of a potentially allergenic food and a confirmation by challenge, unless there was a clear history of a severe systemic reaction. Urticaria was defined as allergic when appearing at least twice in conjunction with a certain food. Infants were regarded as sensitized if they had at least one positive skin prick test reactivity 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 standardized cat, birch and timothy extracts (Soluprick®, ALK, Hørsholm, Denmark) at 6, 12 and 24 months and seven years 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 $\geq$3mm. Circulating IgE antibodies to egg white and

cow's milk were analyzed 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 analyzed at 6, 12 and 24 months of age (UniCap® Pharmacia CAP System™, fx5, Pharmacia Diagnostics). All of the 21 children developing allergy were sensitized, while none of the 31 healthy children were sensitized (Table 1). Nineteen of the allergic children had eczema, 9 asthma, 6 ARC and 3 urticaria during the first seven years of life. Several children developed more than one allergic symptom.

RESULTS

Healthy adults exhibit circulating antibodies to antigens of the intestinal microbiota. To investigate the IgG adaptive immune response to intestinal microbiota in humans, we employed a novel protein microarray containing recombinant protein antigens and cloned from the murine microbiota (24, 25). The antigens were chosen because all had been found previously to be immunogenic in mice (references 24, 25) and IgG seroreactivity to most of them was found in normal human sera in pilot studies. The individual protein and DNA sequences of these antigens were searched against sequences from the described human microbiota in the MetaHit (20), Human Microbiome Project (www.hmpdacc.org) and NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) databases and the percentage of exact amino acid matches (% identity) and percentage of exact and similar (% positives) amino acid matches are listed, along with a putative protein ID and function (Table 2). Each

sequence was also matched to the Phyla, Class, and primary species to which it was tracked. Of the antigens selected for the array, 31 of 38 had >70% homology to sequences from the human gut microbiota, while 37 of 38 had greater than 50%, suggesting that there are evolutionarily conserved antigens or shared microbial colonization between mice and humans. One antigen, rIB20, appears to be a unique sequence as it had no greater than 50% homology to any known protein sequence in the NCBI database. Two of the antigens, rIB2 and rIB5, had high homology sequence matches to highly conserved sequences found in multiple phyla. Counting these overlapping sequences, the antigens on our array represent 8 Bacteroidetes antigens, 24 Firmicute antigens, and 8 Proteobacteria antigens: 7 are involved in metabolic functions, 13 are flagellin/ motility proteins, 6 are transcription/translation machinery, and 12 others are cell surface proteins of various kinds. Thus the array represents a diverse set of antigens from the three most prominent phyla of the human gut microbiota (Table 2). Several of these antigens, particularly Firmicute flagellins, are known immunodominant antigens in Crohn's disease (24).

Sera from healthy adults from Canada, Sweden, and the United States were tested against this panel and the immune response to antigens from the intestinal microbiota was evidenced by the presence of serum IgG reactivity (Fig. 1). Despite individual differences in magnitude of response, a common pattern of response to particular antigens emerged (Fig. 1A). Though not all antigens were recognized by individual sera, there was a significant correlation in reactivity between specific antigens, particularly among Firmicute flagellins, and among four "universal" antigens (Supplemental Fig. 1). In regard to the latter, nearly every adult individual had a strong response to these four antigens: rIB1, rIB10, rIB2 and rIB20 (Fig. 1b, Supplemental Fig.

208 2). Because IgG responses to peptide antigens requires CD4+ T-helper cells to stimulate
209 isotype switching, these data reflect the participation of both T cell and B cell immunity
210 in generating a response to these antigens. Further, these data indicate that even in
211 healthy individuals, there is a normal adaptive immune response to antigens present in
212 the commensal microbiota, including antigens known to be targets in inflammatory
213 disease.

214 **Perturbation of the seroresponse to antigens from the intestinal**
215 **microbiota in individuals with allergic disease.**

216 We next studied allergic individuals, because childhood allergy is associated with
217 alterations of the intestinal microbiota (1, 17, 26). Serum collected 1-week post-partum
218 from 53 Swedish mothers, 30 of which had allergic disease, and 23 who did not, were
219 compared to 40 non-allergic Swedish adults. In contrast to the seroresponse seen in
220 healthy individuals, IgG reactivity in women with allergic disease was significantly lower
221 to each cluster of antigens including the four universal antigens (Fig. 2). Each of the
222 mothers was recruited based on having allergy, or having children at risk for allergy. In
223 post-partum women without allergy, there was still a significant reduction in IgG
224 responses (Fig. 3a) on par with the weak responses seen in atopic individuals, with the
225 single exception of Firmicute flagellin antigens. The reactivity to this group of antigens
226 is equivalent to the reactivity observed in North American adults (Fig. 3B).

227 **Differences in seroresponses to antigens from the intestinal**
228 **microbiota are present in infancy.** While the reactivity to universal antigens was
229 perturbed in the mothers, at 2 years of age the magnitude of reactivity in their children
230 was significantly greater, and compared equivalently to adult controls (Fig. 3A). In
231 contrast, the seroreactivity to the set of Firmicute flagellins at 2y of age in healthy

children was significantly greater than the reactivity observed in the unrelated controls ($p < 0.001$). The magnitude of this response to Firmicute flagellin antigens approaches that seen in adult Crohn's Disease patients to these antigens (Fig. 3B).

Although allergic adults have a lower adaptive IgG response to microbiota antigens, little is known about the development of this response in children. It is possible that the reduced adaptive immune response that correlates with certain immune-mediated diseases in adults could predispose children to the development of disease. To this end, sera from 52 Swedish children were collected at time points from 6 months to 7 years of age and analyzed via protein microarray for serum IgG reactivity to antigens from the gut microbiota. These children all had a family history of allergic disease, and during the course of the study 21 developed allergic disease and 31 remained allergy free (22). Seroresponses to antigens from Bacteroides (Fig. 4a), Firmicute flagellin (Fig. 4b), other Firmicute proteins (Fig. 4c), or Proteobacteria (Fig. 4d), or to the four universal antigens (Fig. 4e-h) were grouped together and compared to cord blood (labeled as 0m, representing antibodies transferred to the child during pregnancy), and then from 6m to 7y of age. Allergic children had significantly lower reactivity to Firmicute (Fig. 4c) and Universal (Fig. 4e-h) antigens at 6m of age, and continued this trend until 7y. This pattern of lower reactivity is even evident in the cord blood (Fig. 4e, g). While serum antibody levels against the majority of antigens on the array were very low at 6m, serum antibodies against the 4 universal antigens were detectable at 6 months of age, and for each of these antigens, allergic children, or those who would develop allergy, had significantly lower seroreactivity than did healthy children. This trend continued throughout early life. Reactivity to non-flagellin antigens from the Firmicute phyla was also significantly higher at 6m in healthy children than in

256 those developing allergy, and continued in this trend, mirroring the pattern observed
257 with reactivity to universal antigens.

258 Many external factors such as breast feeding exclusivity and duration (27, 28),
259 use of antibiotics (29, 30), and probiotic therapies have been proposed to alter the
260 composition of the gut microbiota (31). Though they may alter the composition, in our
261 study we found no significant differences when children in the study were grouped by
262 exclusive breastfeeding during the first three months of life, or probiotic use
263 (Supplemental Figures 3,5). Treatment with antibiotics under age 2 increased reactivity
264 selectively to several of the universal antigens at 24 months of age (Supplemental Figure
265 4), however antibiotic treatment had no effect on the subsequent development of
266 allergy. Only three children were delivered by Caesarean section in the allergic and non-
267 allergic groups, which is too small a number to make valid comparisons of
268 seroreactivity. Detailed comparisons between the allergic and non-allergic children are
269 provided in Table 1.

270

271 **DISCUSSION**

272 Though the composition and development of the microbiota is beginning to be
273 understood (19-21), the interplay of the normal human adaptive immune response and
274 the human microbiota, especially in healthy individuals, remains to be explored. This
275 protein microarray serves as a unique tool to investigate this interplay and begin to
276 define the normal response to antigens in the microbiota. Each of the antigens present
277 on the array was initially cloned as a result of being immunogenic in mice (24, 25). The
278 majority of them had very high homology matches to sequences from the characterized
279 human microbiome, suggesting that these are evolutionarily conserved epitopes. Our

data clearly indicate that individuals with allergic disease have a decreased response to clusters of antigens from the commensal gut microbiota compared to healthy individuals. Furthermore, low IgG responses to certain clusters of microbiota antigens during infancy were associated with allergy development during childhood. To confirm our microarray results, we cross-linked selected antigens to Luminex beads and compared the relative binding signals observed in a fluorescent Luminex bead assay to the values obtained for the same samples from the protein microarray; for each group tested, the same pattern of reactivity and same level of significance was obtained between the protein microarray and the Luminex bead assay (data not shown).

Our results demonstrate that rather than there being a lack of response to the gut microbiota, there instead is activation of the adaptive immune system evidenced by IgG seroreactivity to antigens derived from the microbiota. It has been known for some time that there is normal IgG autoantibody production in healthy individuals (32, 33), and these autoantibodies are directed to multiple different cellular components, both intracellular and extracellular. As such it should be expected that there is also adaptive immune activation directed towards the commensal microbiota. In this study we highlighted four particular antigens as being "universal", in that nearly every individual tested responded to all four of them. Aside from rIB20, the identity of which remains unknown, the other 3 universal antigens all come from proteins involved in transcription and translation. As naturally occurring autoantibodies are likely to play a role in proper immune function, it appears that an active immune response to the microbiota is also involved in normal immune function.

The reduced reactivity to universal antigens observed in allergic children appears to be present in the mothers of allergic children as well. Though allergic children had

304 reduced reactivity to these universal antigens compared to healthy children, at 2 years of
305 age the magnitude of reactivity in all children was greater than that of their mothers at
306 parturition, matching the levels observed in the control group (Fig. 3A). Of note, the
307 seroreactivity to the set of Firmicute flagellins at 2y of age was significantly greater than
308 the reactivity observed in either mothers ($p < 0.0001$ for all cases) or in the unrelated
309 controls ($p < 0.001$ for all cases). The magnitude of this response to Firmicute flagellin
310 antigens approaches that seen in adult CD patients to these antigens (Fig. 3B).
311 Although the magnitude of response in children was higher than that of their mothers or
312 healthy adults, the pattern of antigens to which they respond was the same. The
313 common pattern of seroreactivity in infants contrasts with the succession of varying
314 microbiota colonization as assessed by 16s-DNA that has been observed in African,
315 South American, and North American children before the age of three, when the
316 microbiota begins to resemble that of adults (4, 6, 34). However, this common pattern
317 of reactivity may be coherent with the common functional microbiota that has been
318 revealed by metagenomic studies (20). This succession of bacterial colonization and
319 eventual stabilization may contribute to the heightened response observed from 12 to 24
320 m that is followed by the retreat to more adult-like levels at 7 y of age.

321 The children in this study came from a study of probiotic effectiveness in which
322 newborns at risk for allergic disease received *Lactobacillus reuteri* from birth in an
323 attempt to reduce the incidence of allergy. This same group of children has been shown
324 to have a reduced diversity in the gut microbiota (17), a circumstance also seen in
325 Crohn's disease (35, 36) and T1D (37). A lower adaptive immune response to the gut
326 microbiota can be suggested as a predisposing factor for development of atopy, because
327 all of these children were considered "at-risk" for allergy development. Indeed,

328 comparing the seroreactivity of the mothers of these children to healthy controls (Fig. 2)
329 indicated that as a group, these mothers had lower seroreactivity than healthy controls.
330 When mothers were separated by their own allergy status, allergic mothers comprise the
331 lowest responding cohort. Pregnancy profoundly decreases the richness of the
332 microbiota (38), and thus some of the decrease in reactivity in the mothers could be due
333 to these alterations. However, there remain clear differences in reactivity to antigens
334 from the gut microbiota between allergic and non-allergic mothers.

335 We are colonized during transition out of the birth canal. Newborns are protected
336 during initial exposures to microbes via trans-placental passage of maternal IgG, but
337 must respond on their own after the mother's IgG is metabolized. This initial response is
338 vigorous and is maintained throughout life at lower levels. Perturbed responses to
339 microbiota antigens correlate with development of certain immune-mediated diseases
340 in adults. Taken together, these data are compatible with the concept that a strong
341 adaptive immune response to the microbiota in infancy is protective against immune
342 mediated disease later in life. An appropriate intensity and diversity of microbial
343 stimulation during infancy may be required for adequate development of the adaptive
344 immune system (42). This concept is consistent with the findings of a reduced gut
345 microbiota diversity during infancy preceding development of atopic eczema (17, 39-41)
346 and asthma (18).

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AUTHOR CONTRIBUTIONS

B. Christmann is responsible for the generation and analysis of the microarray data and for manuscript preparation. M. Jenmalm and C. O. Elson assisted in study design, manuscript preparation and data analysis. L.W. Duck cloned the antigens and developed the microarray used in these studies. T. Abrahamsson, C. Bernstein, P. Mannon, G. Berg, B. Björkstén, M. Jenmalm, & C. O. Elson contributed to sample collection and study design.

Table 1. Background factors and other allergic manifestations in children with and without allergic manifestation until seven years of age. Follow-up was performed by research nurses at 1, 3, 6, 12, and 24 months of age and by structured telephone interviews with parents at 2, 4, 5, 8, 10, and 18 months. They asked the parents about infections at each contact. Upper respiratory infection dominated. As indicated in the table the mean of infections was 5.4 and 5.5 during the first and second year of life, respectively. The mean of gastrointestinal infections was 0.3 (sd 0.5) and 0.3 (sd 0.5) in the allergic and non-allergic children, respectively (p=0.78, t-test).

Table 2. Antigens represented on the protein microarray. 38 antigens cloned from the murine cecal microbiota (24, 25) were searched against sequences from the described human microbiota in the MetaHit, HMB, and NCBI databases and the percentage of exact amino acid matches (% identity) and percentage of exact and similar (% positives) amino acid matches are listed, along with a putative protein ID and function. Each sequence was also matched to the Phyla, Class, and primary species in the human microbiota to which it was tracked.

Figure 1. There is a normal human adaptive immune response to antigens from the gut microbiota. Serum from 143 healthy, Caucasian adults in 3 countries (Canada, Sweden, USA) was analyzed on the microarray and IgG reactivity to the antigens was determined. A. Data are expressed as mean +/- SEM to illustrate the pattern of response. B. Data are expressed as box and whiskers (10-90%) of fluorescence intensity for each antigen to illustrate the variance of the response among

individuals. Four antigens, rIB1, rIB2, rIB10, and rIB20 were found to be universally recognized among nearly all individuals.

Figure 2. The human adaptive immune response to antigens from the gut microbiota differs between mothers of at-risk children and controls. Swedish mothers were separated by health status (24 allergic, 30 non-allergic), and the average reactivity to antigens from different phyla or universal antigens were compared to that of healthy controls (n=40). Data are expressed as mean±SEM. ** $p<0.005$, ***$p<0.0005$, ****, $p<0.0001$, Mann-Whitney test.

Figure 3. (A) IgG reactivity to the universal antigens for Swedish mothers was compared to all infants (regardless of allergy) at 24 months, or control, adult Swedes, mean±SEM. (B) IgG reactivity to Firmicute flagellins of healthy, 24 month-old, Swedish infants (n = 31) compared to healthy, Swedish adults (n = 40), healthy, adult, North Americans (n = 54), or adults with CD from the US and Canada (n = 45), mean±SEM. ** $p<0.005$, ***$p<0.0005$, ****, $p<0.0001$, Mann-Whitney test.

Figure 4. Development of the human adaptive immune response to antigens from the gut microbiota begins in infancy and a reduced seroreactivity is observed in children developing allergy. Average fluorescence intensity to antigens from the different clusters (A-D) or the 4 universal antigens (E-H) was compared in sera from 33 healthy Swedish children (control, squares) or 21 who developed eczema (allergic, open circles) over the first 7 years of life, and in the cord blood of their mothers (Om time point; mothers are grouped by the health status of

their child). Data are expressed as mean±SEM. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$, compared to same time point.

FIGURE 1

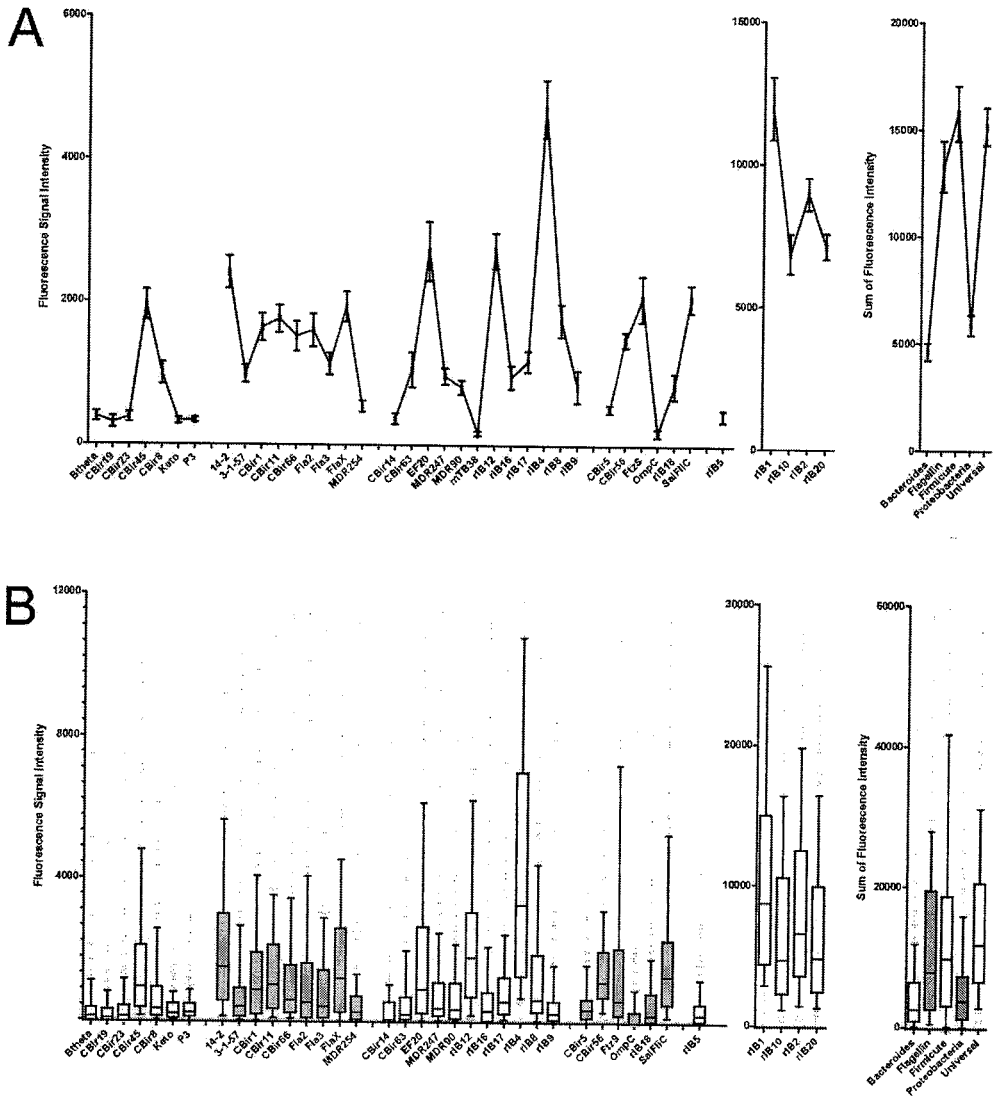

A
Fluorescence Signal Intensity
Sum of Fluorescence Intensity
Bacteroides
Flagellin
Firmicutes
Proteobacteria
Universal
B
Fluorescence Signal Intensity
Sum of Fluorescence Intensity
Bacteroides
Flagellin
Firmicutes
Proteobacteria
Universal

FIGURE 2

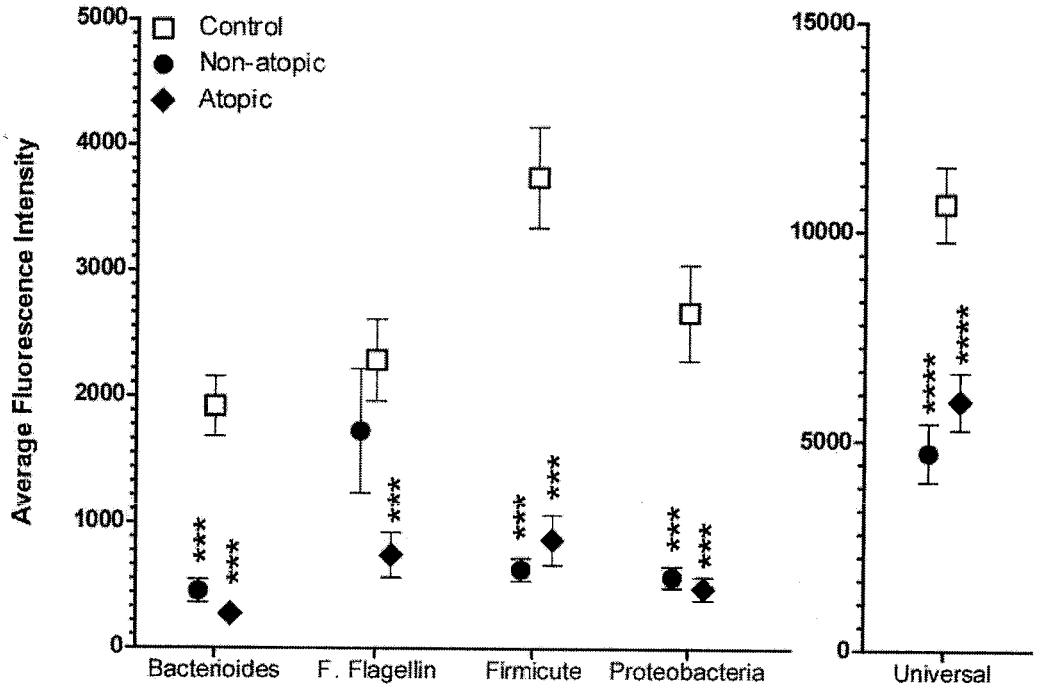

Control
Non-atopic
Atopic
Average Fluorescence Intensity
5000
4000
3000
2000
1000
0
15000
10000
5000
0
***
***
***
***
***
***
****
****
Bacteroides
F. Flagellin
Firmicute
Proteobacteria
Universal

FIGURE 3

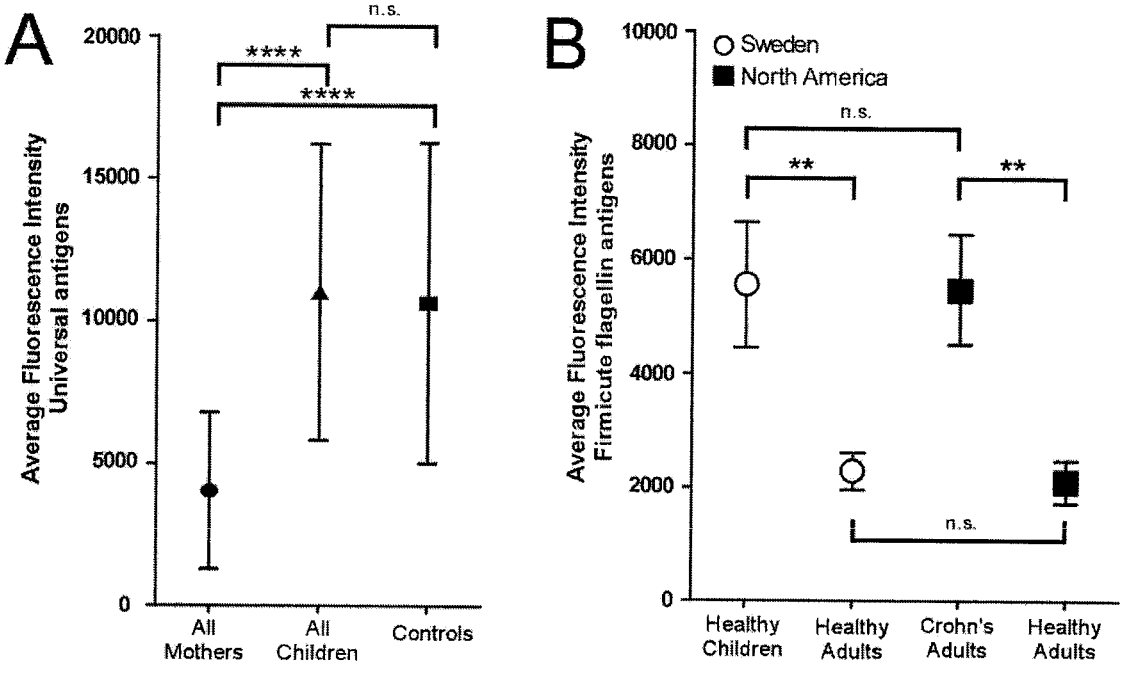

A
Average Fluorescence Intensity
Universal antigens
20000
15000
10000
5000
0
****
****
n.s.
All Mothers
All Children
Controls
B
Average Fluorescence Intensity
Firmicute flagellin antigens
10000
8000
6000
4000
2000
0
Sweden
North America
n.s.
**
**
n.s.
Healthy Children
Healthy Adults
Crohn's Adults
Healthy Adults

FIGURE 4

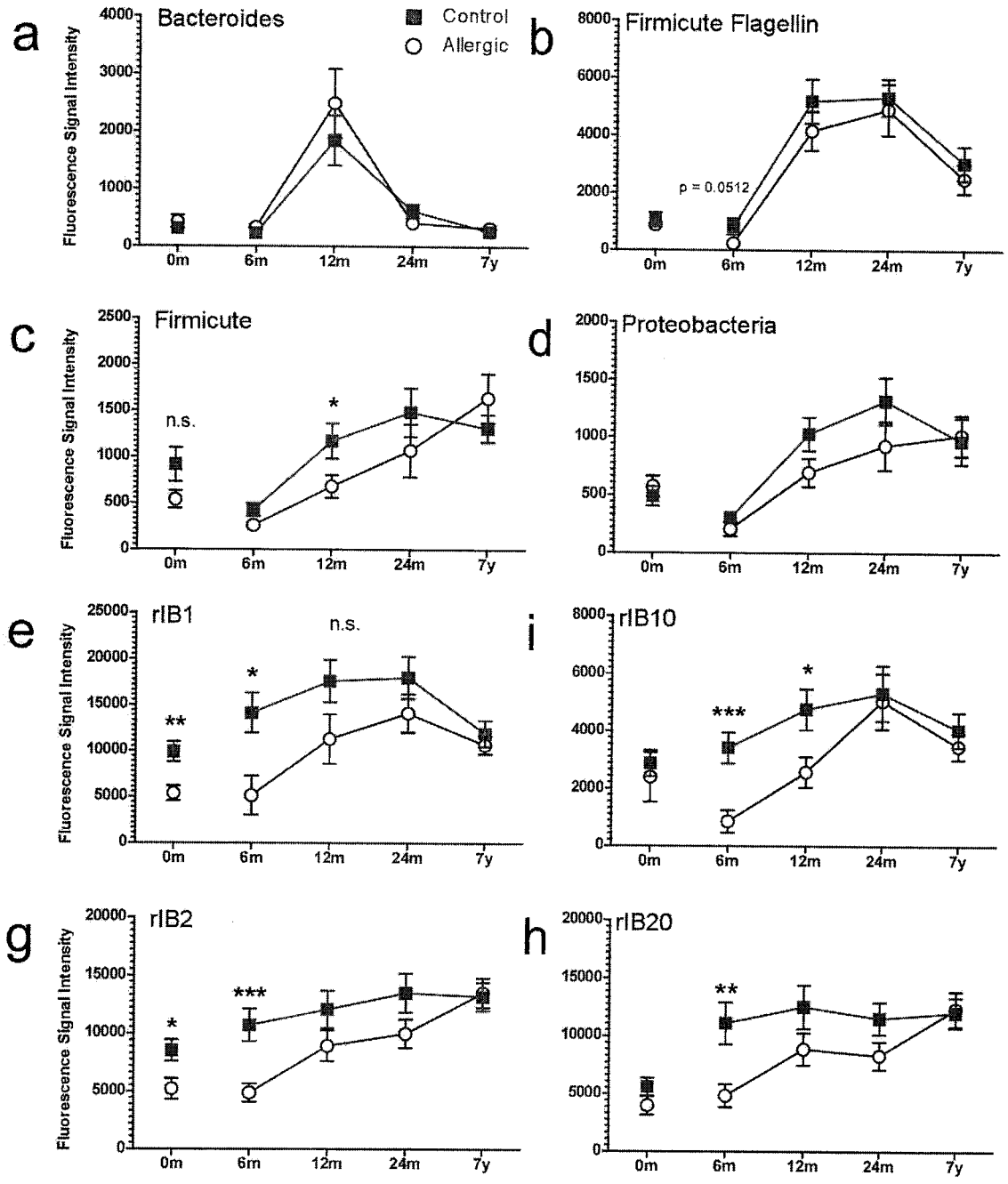

a
Bacteroides
Control
Allergic
Fluorescence Signal Intensity
4000
3000
2000
1000
0
0m
6m
12m
24m
7y
b
Firmicute Flagellin
8000
6000
4000
2000
p = 0.0512
c
Firmicute
2500
n.s.
*
d
Proteobacteria
1500
500
e
rIB1
25000
20000
15000
10000
5000
**
i
rIB10
***
g
rIB2
h
rIB20

Table 1.

	Allergic disease until 7 years		
	Yes	**No**	
	% (n/N)	**% (n/N)**	P*
Probiotic group	38 (8/21)	42 (13/31)	0.78
Boys	52 (11/21)	52 (16/31)	0.96
Older sibling	43 (9/21)	45 (14/31)	0.87
Maternal allergic disease	71(15/21)	90 (28/31)	0.13
Asthma	14 (3/21)	27 (7/31)	0.72
Allergic rhinoconjunctivitis	19 (4/21)	45 (14/31)	0.05
Eczema	27 (6/21)	26 (8/31)	0.83
Food allergy	24 (5/21)	10 (3/31)	0.24
Allergic urticaria	24 (5/21)	7 (2/31)	0.10
Atopic (sensitized to allergens)	38 (8/21)	65 (20/31)	0.06
Caesarean section	14 (3/21)	10 (3/31)	0.68
Breastfeeding (exclusive) at 3 m	76 (16/21)	72 (22/31)	0.68
Breastfeeding (any) at 6 m	76 (16/21)	84 (26/31)	0.50
Breastfeeding (any) at 12 m	10 (2/21)	26 (8/31)	0.17
Parental smoking (prebirth)	5 (1/21)	10 (3/31)	0.64
Furred pets at birth	10 (2/21)	13 (4/31)	1.00
Antibiotics 0-6 m	5 /1/21)	16 (5/31)	0.38
Antibiotics 6-12 m	14 (3/21)	26 (8/31)	0.49
Antibiotics 12-24 m	33 (7/21)	48 (15/31)	0.28
Infections 0-12m mean (sd)	5.4 (2.9)	5.3 (3.0)	0.90
Infections 12-24m mean (sd)	5.5 (3.8)	5.4 (4.2)	0.91
Day-care at 12 months of age	5 (1/21)	7 (2/31)	1.00
Day-care at 24 months of age	71(15/21)	81 (25/31)	0.51
Asthma until 7 y	43 (9/21)	0 (0/31)	<0.001
Allergic rhinitis until 7y	29 (6/21)	0 (0/31)	0.003
Eczema until 7y	91 (19/21)	0 (0/31)	<0.001
Allergic urticaria until 7 y	14 (3/21)	0 (0/31)	0.06
Sensitization until 7y	100 (21/21	0 (0/31)	<0.001

* Chi2 test was employed for categorical variable. Fisher's exact test was used when the expected frequency for any cell was less than five. Student t-test was employed for continuous variables.

TABLE 2

Phylum	Class	Name	Protein ID	Function	Primary Species	% identity	% positives
Bacteroidetes	Bacteroidia	Btheta	CBir28 - hypothetical protein	other/unknown	Bacteroides sp.	70	87
		CBir19	ABC transporter.ATP-binding protein	other/unknown	Bacteroides vulgatus	78	93
		CBir23	elongation factor 1A	transc/transl	Alistepes shahii	85	95
		Cbir45	glycosyl hydrolase	metabolism	Bacteroides eggerthii	59	75
		CBir8	elongation factor Tu	transc/transl	Tannerella sp.	90	94
		Keto	transketolase	metabolism	Bacteroides fragilis	100	100
		P3	HSP90	other/unknown	Bacteroides fragilis	80	80
Firmicute flagellin	Clostridia	14-2	flagellin from 14-2 isolate	motility	Roseburia intestinalis	80	87
		3_1_57	flagellin	motility	Lachnospiraceae	100	100
		CBir1	flagellin	motility	Butyrivibrio fibriosolvens	83	89
		CBir11	flagellin	motility	Roseburia inulinivorans	46	58
		CBir66	flagellin	motility	Roseburia intestinalis	66	83
		Fla 2	flagellin 2 from A4 isolate	motility	Roseburia intestinalis	72	80
		Fla 3	flagellin 3 from A4 isolate	motility	Roseburia inulinivorans	81	91
		Fla X	flagellin	motility	Roseburia inulinivorans	56	70
		MDR254	flagellin	motility	Flavonifractor plautii	73	78
Firmicutes	Bacilli	CBir14	GAPDH	metabolism	Lactobacillus salivarius	90	93
		EF20	Sal A	other/unknown	Enterococcus faecalis	100	100
	Clostridia	CBir63	Ig-like surface protein	other/unknown	Roseburia intestinalis	47	64
		MDR247	adenine deaminase	metabolism	Clostridium bolteae	54	76
		MDR90	collagen adhesion protein	other/unknown	Roseburia intestinalis	66	77
		rIB12	homoserine dehydrogenase	metabolism	Flavonifractor plautii	60	76
		rIB16	pyruvate synthase	metabolism	Flavonifractor plautii	74	86
		rIB17	NlpC/P60	other/unknown	Flavonifractor plautii	39	56
		rIB4	relaxase	transc/transl	Clostridium asparagiforme	63	72
		rIB8	glycosyltransferase	metabolism	Lachnospiraceae 3-1-57	59	75
		rIB9	methyl-accepting chemotaxis protein	motility	Roseburia intestinalis	45	63
Proteobacteria	Delta/Epsilon	CBir5	methyl-accepting chemotaxis protein	motility	Helicobacter cinaedi	63	74
		CBir56	methyl-accepting chemotaxis protein	motility	Helicobacter canadensis	60	71
	Gamma	FtsZ	FtsZ protein - homologue of tubulin (putative pANCA)	other/unknown	Escherichia coli	100	100
		OmpC	OmpC from UNC *E. coli*	other/unknown	Escherichia coli	100	100
		SalFliC	Salomonella dublin Flagellin	motility	Salmonella dublin	100	100
	Epsilon	rIB18	surface array protein	other/unknown	Campylobacter showae	30	53
Firm & Prot		rIB5	hypothetical protein - cytoplasmic	other/unknown	Erysipelotrichaceae 3-1-53	37	60
Firmicutes	Clostridia	rIB1	RecN	transc/transl	Clostridium citroniae	80	82
		rIB10	Nucleotidyltransferase/ hypothetical	transc/transl	Roseburia intestinalis	48	70
Proteobacteria	Beta	rIB20	ABC transporter	other/unknown	Verminephrobacter eiseniae	<50	<50
Firm & Bact		rIB2	SAM domain protein	transc/transl	Blautia hansenii	61	77

Supplemental Figure 1. Positive IgG seroreactivity to antigens from the gut microbiota correlates to seroreactivity to other antigens. Sera from 143 healthy, Caucasian adults in 3 countries (Canada, Sweden, USA) were analyzed on the microarray and IgG reactivity to the antigens was determined (Figure 1). Spearman's rho (r) correlation coefficient for each antigen was calculated against each other antigen on the array, and significant ($p < 0.05$) correlation coefficients are displayed. Blue coloring intensity indicates negative correlations, red coloring intensity indicates positive correlations, white indicates no significant correlation. There was a high degree of significant correlation among the Firmicute flagellins (orange labels), and also among the four "universal" antigens (green labels). Interestingly, there were also strong correlations among sequence and structurally unrelated antigens such as Fla2 (flagellin from *Lachnospiraceae* A4) and rIB8 (glycosyltransferase from *Lachnospiraceae*), or rIB20 (unknown) and rIB4 (relaxase from *Clostridia*). Negative correlations exist between some of the universal antigens and B-theta, a Bacteroides antigen, or OmpC, a Proteobacteria antigen.

Supplemental Figure 2. IgG seroreactivity to four "universal" antigens from the gut microbiota in Caucasian adults. Sera from all 341 Caucasian adults in 3 countries (Canada, Sweden, USA) were analyzed on the microarray and the fluorescent signal intensity for four "universal" antigens was plotted for each individual. Median, mean, and standard deviation (raw values and log 10) are listed below each antigen. 99% (336/341) responded to at least two of these antigens, while 96% (327/341) responded to at least three, and 82% (280/341) responded to all four. In this case, "response" is defined as a fluorescence signal intensity greater than 2 Standard

Deviations below the logarithmic mean (223.6, dashed horizontal line). 328 individuals recognized rIB1, 312 recognized rIB10, 318 recognized rIB2, and 324 recognized rIB20.

Supplemental Figure 3. The effect of formula feeding on the development of the human adaptive immune response to antigens from the gut microbiota. Sera from Swedish children were grouped according to exclusive breast feeding at 3 months (squares) or introduction of formula (circles, NBF), and the sum of fluorescence intensity to antigens from a particular grouping was graphed from 6 months to 7 years of age as mean±SEM. No significant differences were observed among any group of antigens at any age, suggesting that the introduction of formula is not affecting the response.

Supplemental Figure 4. The influence of early antibiotics on seroreactivity to the microbiota.

Children who received antibiotics under age 2 years were compared to those who did not. The antibiotics used included ampicillin, heracillin, cephalosporin, sulfa, and macrolides.

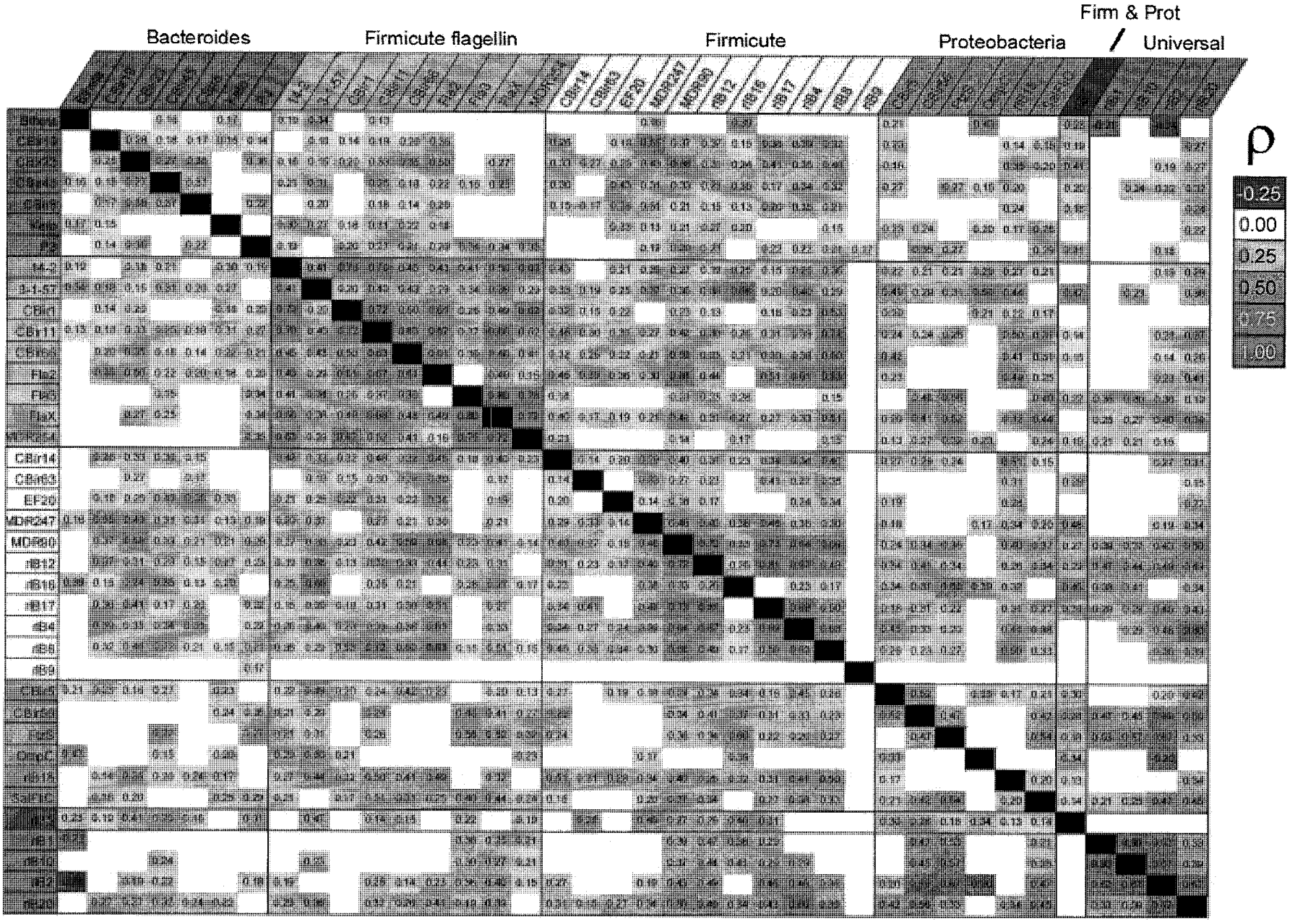
Top panel. Children who did not develop allergy. At two years, there are significant differences in healthy children treated with antibiotics (n=18) in seroreactivity to 3 of the universal antigens. Children who did not receive antibiotics (n=14) had a lower response than children who had been treated with some form of antibiotics.

Bottom panel. All children, regardless of allergy. Children treated with antibiotics (n=26) had a higher response to three of the universal antigens than did children not treated with antibiotics(n=28).

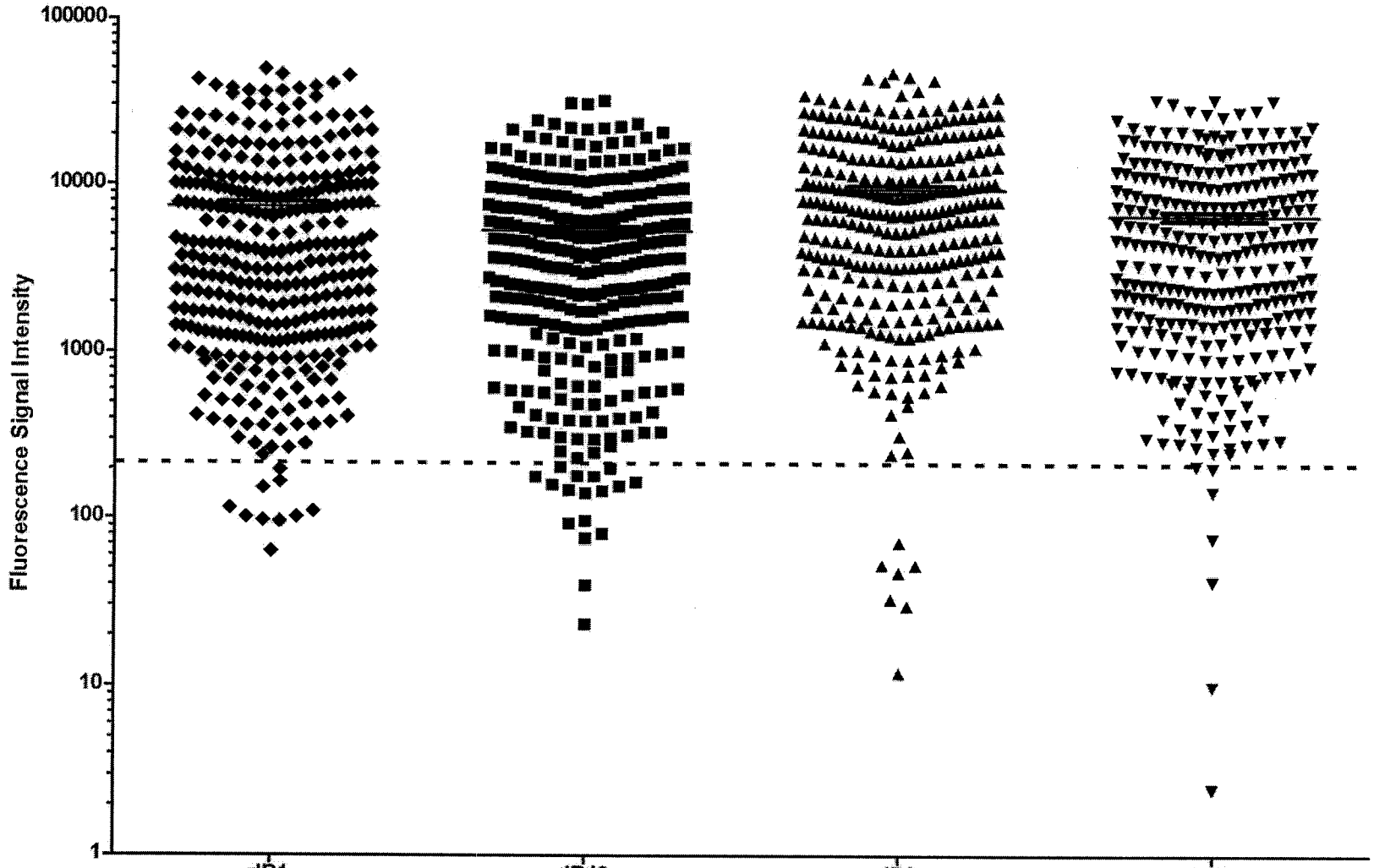
* $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$

Supplemental Figure 5. The effect of probiotic treatment on the development of the human adaptive immune response to antigens from the gut microbiota. Sera from Swedish children were grouped according to treatment with probiotics (squares) or untreated (circles), and the sum of fluorescence intensity to antigens from a particular grouping was graphed from 6 months to 7 years of age as mean±SEM. Mothers received *Lactobacillus reuteri* prior to birth, and neonates were supplemented with *L. reuteri* through 12 months of age. Interestingly, at 24 months of age, there are significant reductions ($p < 0.05$) in the probiotic group compared to the control group. The rest of our data would suggest that reduced reactivity to antigens from the gut microbiota is associated with allergy development, yet probiotic treatment reduced IgE-associated eczema [31]. However, these differences do not correlate with the development of eczema, the effect appears to be localized to 24 months of age, and the differences are not present at 6 and 12 months. It is at these early time points where there are significant differences in seroreactivity that are associated with allergy development (Figure 2).

Supplemental Figure 1.

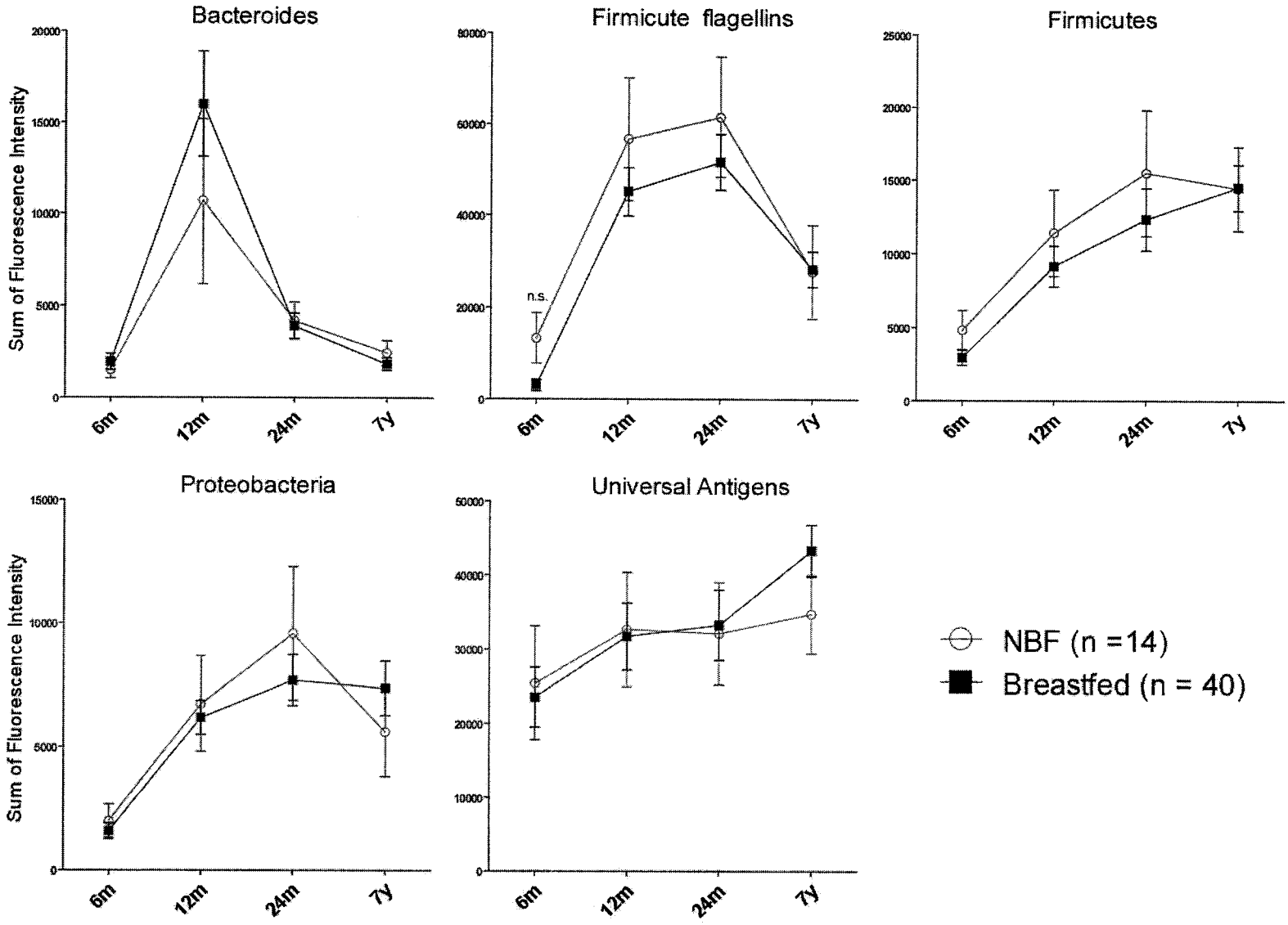


Supplemental Figure 2.



	rIB1	rIB10	rIB2	rIB20
median	3523.5	3414.375	6387.875	3779.375
mean	7402.636	5330.71	9582.667	6701.988
standard deviation	9397.233	5857.011	9786.116	7302.327
log 10 median	3.54504	3.55458	3.822708	3.63271
log 10 mean	3.529645	3.455427	3.720601	3.54637
log 10standard deviation	0.597812	0.583061	0.621989	0.594864

Supplemental Figure 3.



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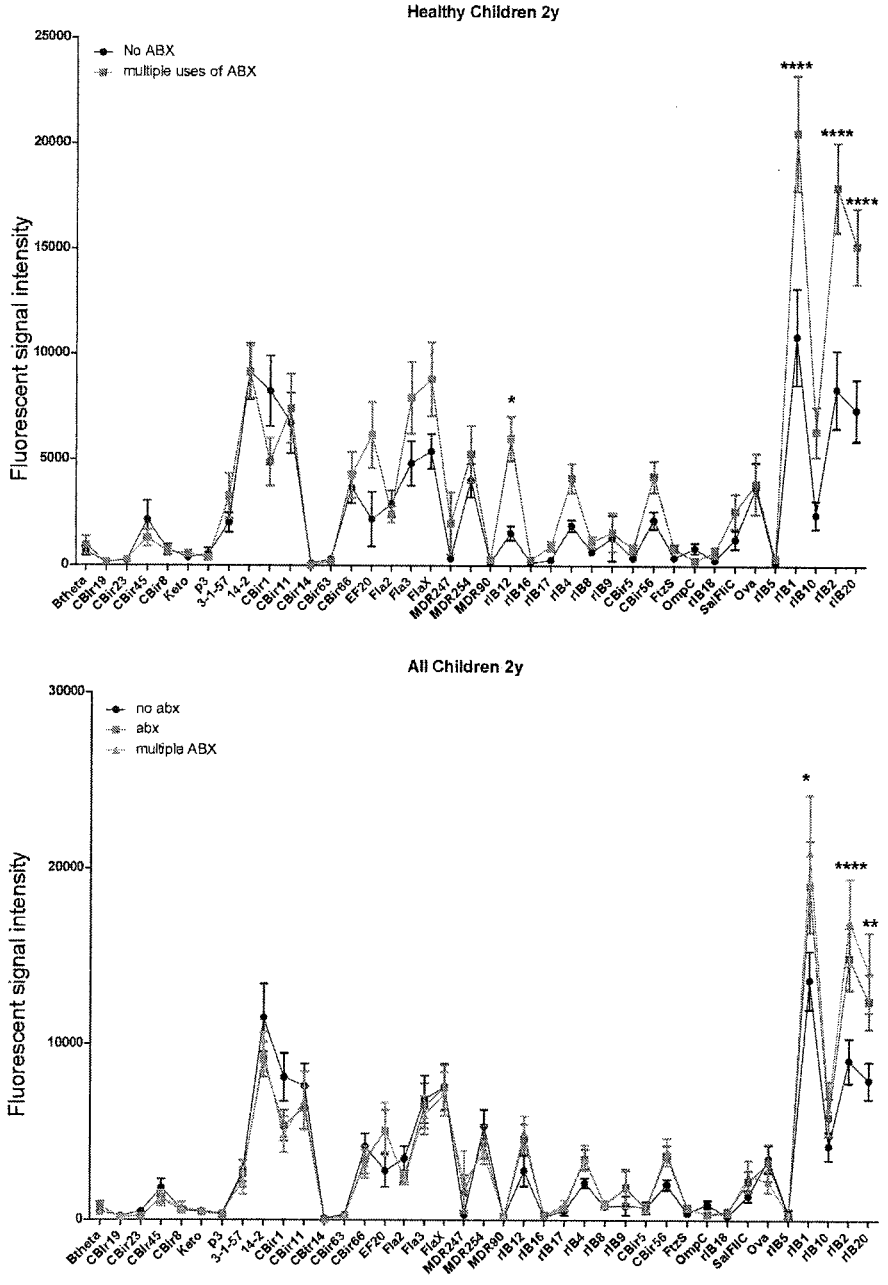

Supplemental Figure 5.