Fecal microbiome and food allergy in pediatric atopic dermatitis: a cross-sectional pilot study

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ABSTRACT

Background

Microbial exposure might be important in the development of atopic disease. Atopic diseases have been associated with specific characteristics of the intestinal microbiome. The link between intestinal microbiota and food allergy has rarely been studied and the gold standard for diagnosing food allergy (double blind placebo controlled food challenge (DBPCFC)) has seldom been used. We aimed to distinguish fecal microbial signatures for food allergy in children with AD.

Methods

Pediatric patients with AD, with and without food allergy were included in this cross-sectional observational pilot study. AD was diagnosed according to the UK Working Party criteria. Food allergy was defined as a positive DBPCFC or convincing clinical history in combination with sensitization to the relevant food allergen. Fecal samples were analyzed using 16S rRNA microbial analysis. Microbial signature species discriminating between presence and absence of food allergy were selected with elastic net regression.

Results

82 children with AD (39 girls, median age 2.5 years old) of which 20 were diagnosed with food allergy provided fecal samples. Food allergy to peanut and cow's milk was the most common. Within children with AD, six bacterial species from the fecal microbiome were identified that when combined discriminate between children with and without food allergy: *Bifidobacterium breve, Bifidobacterium pseudocatenulatum, Bifidobacterium adolescentis, Escherichia coli, Faecalibacterium prausnitzii* and *Akkermansia muciniphila (*AUC 0.83, sensitivity 0.77, specificity 0.80).

Conclusions

In this pilot study, we identified a microbial signature in children with AD that discriminates between absence and presence of food allergy. Future studies are needed to confirm our findings.

INTRODUCTION

The worldwide prevalence of atopic disease has been increasing in recent decades.[1] There is no clear reason for this observed increase in prevalence, but reduced early-life exposure to different microbes is thought to be a contributing factor.[2-4] Microbial colonization of the human intestine during infancy is important for the maturation of the immune system.[5, 6] Intestinal microbiota can regulate metabolic and inflammatory responses and also modulate changes in the intestinal barrier. Several studies have shown associations between intestinal microbiota and subsequent development of atopic disease, including atopic dermatitis, asthma or rhinitis. However, few studies have investigated the link between specific patterns of intestinal microbiota and food allergy. Furthermore, the gold standard for diagnosing food allergy (double blind placebo controlled food challenge (DBPCFC)) has rarely been used.[7]

The microbiome can be considered a complex ecosystem where various species interact and group-based correlations have been identified.[5] Therefore the symbiosis of the different bacterial species and their patterns should be taken into account in data analysis. To be able to identify individual species and take the existing structures within the microbiome into account, advanced statistical modelling techniques are needed. Furthermore, assessment of microbial diversity with molecular sequencing techniques, as opposed to culture-based techniques, reveals greater diversity and has shown the importance of uncultured species.[8]

We hypothesize that children have distinct microbial patterns in their fecal microbiome that are associated with a clinical diagnosis of food allergy. In this cross-sectional pilot study, we aimed to identify microbial species in children with AD, using 16S rRNA microbial analysis followed by statistical elastic net regression approaches.

METHODS

Study design and study participants

Children with AD who were treated in the outpatient clinic of the Wilhelmina Children's Hospital of the University Medical Center Utrecht participated in this cross-sectional pilot study. Inclusion criteria were: diagnosis of AD, age between 0 and 18 years, parental ability to answer Dutch questionnaires and the availability of a fecal sample for microbiome analysis. All study participants participated in a randomized controlled trial that compares shared medical appointments with individual consultations (ISRCTN08506572). The medical ethical committee of the University Medical Center Utrecht approved the study and written informed consent was obtained from all participants. Clinical history

and serum samples were taken on the same day, fecal samples were provided within the next days and a DBPCFC was planned within months.

Assessment of AD and food allergy

AD was diagnosed according to the criteria of Williams et al.[9] AD severity was estimated using the self-administered eczema area and severity index (SA-EASI) by the research nurse.[10] Sensitization was determined by measurement of specific IgE against common food allergens (hen's egg, cow's milk, peanut, hazelnut, fish, wheat, soy). Both total and specific IgE were measured according to manufacturer's protocol (Phadia, Uppsala, Sweden). Diagnosis of asthma and allergic rhinitis was based on clinical history.

Food allergy was defined as a positive double blind placebo-controlled food challenge (DBPCFC) or convincing clinical history in combination with sensitization to that specific food or in case of peanut allergy, a sensitization to Ara h 2 above the defined cut off level in our clinic (5.17 kU/L).[11] A convincing clinical history was defined as a reported Type I allergic reaction with acute symptoms within 2 hours after ingestion of the food. DBPCFC was considered positive and terminated when persistent objective symptoms occurred (e.g. vomiting, generalized urticaria, wheezing or a significant drop in blood pressure) or after subjective symptoms (oral allergy symptoms, nausea, abdominal discomfort) on three subsequent doses or a severe subjective symptom (abdominal pain/nausea with discomfort) lasting for more than 45 minutes, according to the international protocol.[12] Late reactions were assessed using follow-up by telephone the next day.

Fecal samples

Children collected fecal samples at home and sent the samples to the laboratory using the regular postal service. The samples were aliquoted and frozen at -20^{0}C until further processing.

Fecal DNA isolation

Approximately 150 mg of fecal material was directly transferred to the DNA isolation plate. Then 0.5 mL phenol pH8.0 (Phenol solution, catalogue P4557, Sigma-Aldrich, St Louis, MO) was added and the samples were mechanically disrupted by bead beating 2 times 3 minutes with a 96-well plate Beadbeater (Biospec Products, Bartlesville). Samples were centrifuged at 1880 rcf (4000rpm) for 10 minutes to separate the aqueous and phenolic phases. The aqueous phase was transferred to a 96-well plate and DNA was purified with the AGOWA mag Mini DNA Isolation Kit (AGOWA, LGC genomics, Berlin, Germany) in accordance with the manufacturer's recommendations. After elution, the total bacterial load in each sample was assessed by quantitative PCR using a universal bacterial primer-probe set.[13]

16S rRNA Illumina sequencing

Analysis of the fecal microbiome composition was performed by mass sequencing of the V4 hypervariable region of the 16S rRNA gene on the Illumina MiSeq sequencer (Illumina, San Diego, CA). Barcoded DNA fragments spanning the Archaeal and Bacterial V4 hypervariable region were amplified with a standardizing level of template DNA (100pg) to prevent over-amplification. These amplicons, generated using adapted primers 533F and 806R, were bi-directionally sequenced using the MiSeq system.[14] Pre-processing and classification of sequences was performed using modules implemented in the Mothur V.1.20.0 software platform.[15] The relative abundance of unique sequences was calculated for every fecal sample. The dataset was transformed using zero mean unit variance transformation for subsequent statistical analyses. The V4 amplicon of the 16S rRNA encoding gene allows for discrimination of several Bifidobacterial species, but not all.[16] Therefore, relevant sequences were blasted in the Ribosomal Database Platform (RDP) to determine a more accurate species level. Shannon diversity indices were calculated to describe the microbial diversity.

Statistical analysis

Descriptive statistics were used to describe patient characteristics. Non-parametric tests were used to compare the groups without and with a confirmed food allergy.

Elastic net regression

Bacterial signature species discriminating between absence and presence of food allergy were selected using elastic net regression. This is a statistical machine learning approach, applicable to large scale, structured and higher dimensional data. The method is regularization-based and combines the advantages of LASSO regression (sparsity, retaining the feature selection property of reducing coefficients to exact zero values provided by LASSO) and ridge regression (smoothness, tendency of shrinking coefficients to small values for correlated trending towards each other).[17, 18] All present species and the correlations between them are taken into account, which allows for the identification of patterns of species rather than individual species.[19] Using elastic net regression, it is not possible to correct for other confounding factors which is common in other types of regression analyses used in medical statistics.[19]

Randomization test and ROC/AUC

A randomization test was conducted to test the statistical validity of the results obtained with elastic net regression. Receiver-Operating-Characteristics/Area-Under-Curve (ROC/AUC) scores were generated multiple times after randomly reshuffling the food allergy diagnoses, while keeping the corresponding microbial profiles intact.[20] The dataset was cross-validated by randomly hiding 30% of the children from the model and evaluating

the prediction quality on that group. The predictive accuracy of the classification model was measured with the ROC/AUC score, using a critical value of 0.05.

SPSS (version 22; IBM, Armonk, NY) was used for descriptive data analysis. GraphPad Prism (version 6.01; GraphPad Software, La Jolla, CA) was used for providing graphs and figures. All other statistical analyses were performed using numerical Python (version 2.7, Python Software Foundation, https://www.python.org).

RESULTS

Atopic dermatitis and food allergy

We included 82 children in this cross-sectional pilot study. There were no significant differences regarding sex or age between the children who were included and those who were not (data not shown). All 82 children were diagnosed with AD, 62 children had no food allergy (AD+FA-) and 20 children had a confirmed food allergy (AD+FA+) (table 1).

Of the 62 children without food allergy, almost half were sensitized to common food allergens without having symptoms of food allergy after ingestions. Among the 20 children with a food allergy, peanut allergy and cow's milk allergy were the most common (table 2). Multiple food allergies were found in two children. On average, a DBPCFC was performed within 10 months of providing the fecal samples (minimum 1 month, maximum 27 months).

Table 1. Patient characteristics

	No food allergy (AD+FA-) (n=62)	Confirmed food allergy (n=20) (AD+FA-)	p-value
Age in years [median (IQR)]	3.0 (5)	2.2 (5.5)	0.606
Female [n (%)]	32 (52%)	7 (35%)	0.196
SAEASI [median (IQR)]	29 (32)	46 (42)	0.283
TARC (pg/ml) [median (IQR)]	1243 (2247)	2251 (5613)	0.013
Total IgE (kU/L) [median (IQR)]	66 (281)	564 (2912)	<0.001
Sensitization to any food allergen [n (%)]	27 (43%)	20 (100%)	<0.001
Elimination diet for any food [n (%)]	19 (31%)	20 (100%)	<0.001
Diagnosed with asthma [n (%)]	18 (29%)	5 (25%)	0.727
Diagnosed with rhinoconjunctivitis [n (%)]	15 (24%)	5 (25%)	0.942

Data is shown with median and interquartile range or number and percentage. AD = atopic dermatitis, FA = food allergy, SAEASI = self-administered eczema area and severity index scored by research nurse, TARC = thymus and activation regulated chemokine.

Table 2. Children with confirmed food allergies

Food allergy	Confirmed	With DBPCFC*	Obvious clinical history only	Predicted based on elevated sIgE to arah2 > 5.17 KU/L
	n(%)	n(%)	n(%)	n(%) [11]
Peanut	8 (40%)	3 (15%)	1 (5%)	4 (20%)
Hazelnut	1 (5%)	1 (5%)		
Cow's milk	8 (40%)	6 (30%)	2 (10%)	
Hen's egg	4 (20%)	3 (15%)	1 (5%)	
Other nuts	2 (10%)		1 (5%) cashew nut 1 (5%) pistachio	
Soy	0			
Fish / shrimp	0			
Total	20 (100%)			

Multiple food allergies result in multiple entries. One patient has a confirmed food allergy for cow's milk, peanut and hen's egg, another patient has a confirmed allergy for hazelnut and hen's egg.
*including no late reactions

Sequence and microbiota characteristics

A total of 2.609.478 high quality sequences were obtained (mean=27.182, range=5.825 to 105.404 of sequences per sample) that could be assigned to 12 different phyla and 1.000 unique sequences. The most predominant phyla based on mean relative abundance were Firmicutes (47%), Actinobacteria (32%), Bacteroidetes (9%), Proteobacteria (8%) and Verrucomicrobia (2%), which is characteristic for the gut microbiome of children.[21] Predominant families were Bifidobacteriaceae (28%), Lachnospiraceae (27%), Ruminococcaceae (10%), Enterobacteriaceae (5%), Streptococcaceae (4%) and Coriobacteriaceae (3.5%). Median Shannon diversity indices calculated for the group of children with and without a food allergy were 3.61 (IQR 1.16) and 3.93 (IQR 1.09), respectively (p=0.430).

Identification of microbial biomarkers related to food allergy

We identified six microbial species from four families that together discriminate between the absence and presence of food allergy in children with AD: *Bifidobacterium breve, Bifidobacterium pseudocatenulatum, Bifidobacterium adolescentis* (Bifidobacteriaceae), *Escherichia coli* (Enterobacteriaceae), *Faecalibacterium prausnitzii* (Ruminococcaceae) and *Akkermansia muciniphila* (Verrucomicrobiaceae). On the species level, *Bifidobacterium breve/longum* and *Bifidobacterium pseudocatenulatum/ catenulatum/ gallicum/ kashiwanohense* could not be distinguished after additional blasting in the RDP database, and are referred to as *Bifidobacterium breve* and *Bifidobacterium pseudocatenulatum* throughout the manuscript.

Figure 1 shows the relative abundance of the six identified signature species. The fecal microbiome of children with AD and food allergy harbored relatively more *E.coli* and *B.pseudocatenulatum*, and less *B.breve, B.adolescentis, F.prausnitzii,* and *A.muciniphila*, compared to children with AD without food allergy. The randomization test indicates that the combination of these six species is significantly different between the two groups (p = 0.001), even though the relative abundance of some single species may seem similar on a group level (figure 1). Different relative contributions from the single species towards the total distinctive properties are distinguished, with a larger influence of *B.breve*, *B.adolescentis* and *F.prausnitzii* compared to *B.pseudocatenulatum*, *E.coli* and *A.muciniphila*, expressed as importance indices based on the elastic net regression (figure 2). The overall predictive accuracy of the classification model (area under the curve) is 0.83 (figure 3), with a sensitivity of 0.77 and a specificity of 0.80. Supplementary figures S1 to S3 show the relative abundance of the signature species, the distribution of the 30 most abundant species and the individual distribution of the signature species.

Figure 1. Relative abundance of the microbial signature species in children with AD, without and with a confirmed food allergy

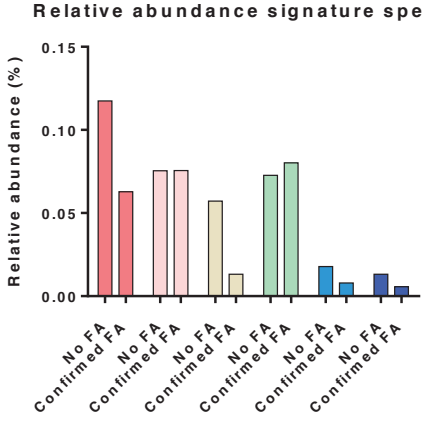
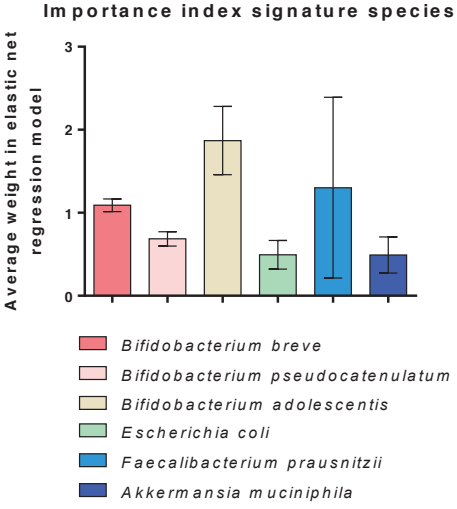


Figure 2. Importance index for signature species in the elastic net regression model



DISCUSSION

We analyzed the fecal microbiome of children with AD with or without a concomitant food allergy and found that a combination of six microbial species, including *E.coli*, *F.prausnitzii, A.muciniphila* and three types of *Bifidobacteria*, discriminates between the presence and absence of food allergy in children with AD (p = 0.001). The fecal

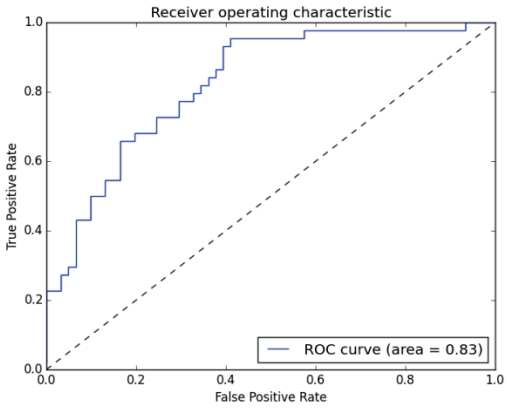


Figure 3. Receiver operating characteristic (ROC) curve of the elastic net regression model

microbiome of children with AD and food allergy harbored relatively more *E.coli* and *B.pseudocatenulatum* and less *B.breve*, *B.adolescentis*, *F.prausnitzii* and *A.muciniphila*, compared to children with AD without food allergy. We found no differences in microbial diversity (according to Shannon index) between the children with AD, with and without food allergy.

This is the first pilot study that identifies microbial signatures specific for food allergy in a group of children with AD using 16S rRNA sequencing techniques generating unique sequences, followed by statistical machine learning approaches. Previous studies mainly used culture-based techniques to analyze the intestinal microbiome or used 16S rRNA sequencing techniques, but subsequently simplified the data in the analysis stage by focusing on key groups of species or analyzing the data on family or genus level. However, this approach leads to less detailed information. For example, the identification of *B.pseudocatenulatum*, *B.breve* and *B.adolescentis* would not have been possible when analyzing the data on a family level. Furthermore, the elastic net regression model takes group-based species interactions into account. Since interactions between species in the gut microbiome occur, this approach may lead to biologically more reliable results compared to other statistical regression approaches.[22]

Our study demonstrates that children with AD and a food allergy had significantly less *Faecalibacterium prausnitzii* and less *Akkermansia muciniphila* compared to children with AD without a food allergy. *F. prausnitzii* and *A. muciniphila* have been gaining interest more recently because of their immune-modulatory properties and possible role in mucosal tolerance. *F. prausnitzii* is the most abundant species in the human intestinal microbiome. Its decreased abundance has been associated with several diseases, including allergic disease and AD. [23-25] *F.prausnitzii* is the main producer of butyrate in the colon, an energy source for colonocytes with important anti-inflammatory effects. It also secretes anti-inflammatory molecules that directly modulate the host immune system, stimulates IL10 producing regulatory T cells and is involved in the balance between

effector and regulatory T cells. [26, 27] *A. muciniphila* is also involved in immunological homeostasis of the gut mucosa and gut barrier function, via an outer membrane protein that stimulates IL10 production.[28]

Bifidobacteria and *E.coli* have been associated with food allergy and AD in other studies.[29] Less *Bifidobacteria* in the feces of children with a confirmed cow's milk allergy has been reported.[30] Cow's milk allergy was a common food allergy in our study population, so it is possible that our results regarding *Bifidobacterium breve* and *Bifidobacterium adolescentis* are mainly contributed by the cow's milk allergic children. Furthermore, we found increased relative abundance of *Escherichia coli* in the food allergic group. *E. coli* has previously been associated with diagnosis of AD, with increasing numbers of *E. coli* further increasing this risk.[31] The children in our study were all diagnosed with AD with varying severity. However, the higher TARC levels in the food allergic group suggest increased AD severity compared to the non-allergic group. This raises the possibility that the selected biomarkers also correlate with AD severity, which fits with the observation that prevalence of food allergy is higher in children with more severe disease.[32]

All microbial species resulting from our analysis have previously been correlated with atopic disease in other studies. This might raise the question whether we are looking at a food allergy specific microbial profile or a profile that is related to atopic diseases in general, as most of these children have or will develop other comorbidities within the atopic syndrome. Atopic disease has been defined differently in previous studies. In our study, all children were clinically diagnosed with AD and in addition asthma and allergic rhinitis was confirmed or ruled out based on clinical history. Food allergy was diagnosed based on DCPCFC in the majority of patients. Post-hoc analyses showed no significant differences in the presence of other atopic diagnoses between the groups with and without food allergy, suggesting that the identified species are indicating food allergy rather than general atopy.

Our study supports the hypothesis that within children with AD the intestinal microbiome differs between children with and without food allergy. Intestinal microbiota regulate the development of a diverse range of T-cell functions, such as Th17, Th1, Th2 and regulatory T cells and modulate innate lymphoid cells.[33, 34] By modifying the response of the gut-associated lymphoid tissues, intestinal microbiota may influence the development of oral tolerance.[35] A recent study in humans showed that delayed colonization with Bacteroidetes is associated with a poorly developed Th1 response, which is important in immune tolerance.[36] It is also possible that disruption of the gut microbiome alters the gut epithelial integrity, thereby increasing the risk of allergic sensitization through direct uptake of allergens.[7] However, the exact mechanisms through which the intestinal microbiome influences food allergy are not elucidated yet. Furthermore, it is not clear whether a change in microbiome precedes or follows the development of food allergy.

Long-term dietary intake affects gut microbiome composition, together with host genetics, age, medication, and general lifestyle.[37] Our study population consumed a Western diet. In addition, established food allergies lead to an elimination diet, where the specific food allergen is excluded from the general diet. We cannot exclude that an elimination diet where one food is excluded from the diet also leads to detectable changes in fecal microbial composition, as has been demonstrated with increased consumption of specific foods. [38] However, in our study one third of children in the non-food allergic group also reported an elimination diet for a specific food because of various reasons. Furthermore, dietary intake varies according to personal preference. Therefore it is unlikely that the observed microbial differences are solely attributed to differences in the consumed diet. Besides a self-reported elimination diet, dietary intake was not further assessed in this study because it is very difficult to assess this accurately.

A limitation of our cross-sectional study is the heterogeneity of the study population. All the children in our study were diagnosed with AD, with varying severity, age and different food allergies, and no healthy controls were included. As expected in children, cow's milk allergy and peanut allergy were the most common food allergies in our study population, so it is possible that our results are influenced by the contribution of these food allergies. It is also plausible that distinct microbes are associated with different food allergies.[29] Due to a lack of statistical power, we were unable to select signature species for specific food allergies. Variables that are known to influence the gut microbiome, such as use of antibiotics, delivery via caesarian-section, or breast feeding, were not assessed in this study.[7] Furthermore, because of the time between the acquisition of the fecal sample and the DBPCFC, transient food allergies could have resulted in the misclassification of some children with cow's milk and hen's egg allergy.

Our findings are based on a study population of children with AD from an academic center. Identifying the microbes that are related to food allergy may help in the development of future interventions. However, future studies are needed to confirm our findings in the community population, preferably with prospective study designs using well-defined patient populations to further explore the potential of fecal microbial colonization patterns associated with specific food allergies in children with AD. Furthermore, future studies should be of sufficient size to allow for stratification of different food allergies and include additional control groups: children with food allergy without AD, children with severe 'extrinsic' AD without food allergy.

CONCLUSION

In this pilot study, we identified a microbial signature in children with AD that discriminates between absence and presence of food allergy. Future studies are needed to confirm our findings.

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SUPPLEMENTARY MATERIAL

Figure S1. Relative abundance of the six signature species in children with AD, without and with food allergy

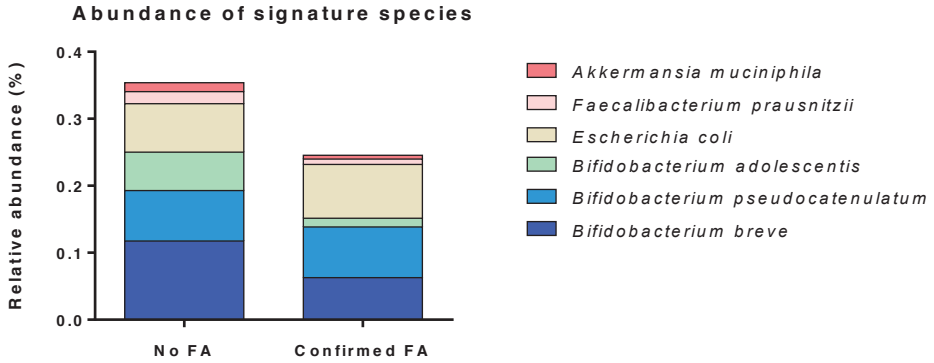

Figure S2. Distribution of 30 most abundant microbial species in children with AD, without and with food allergy

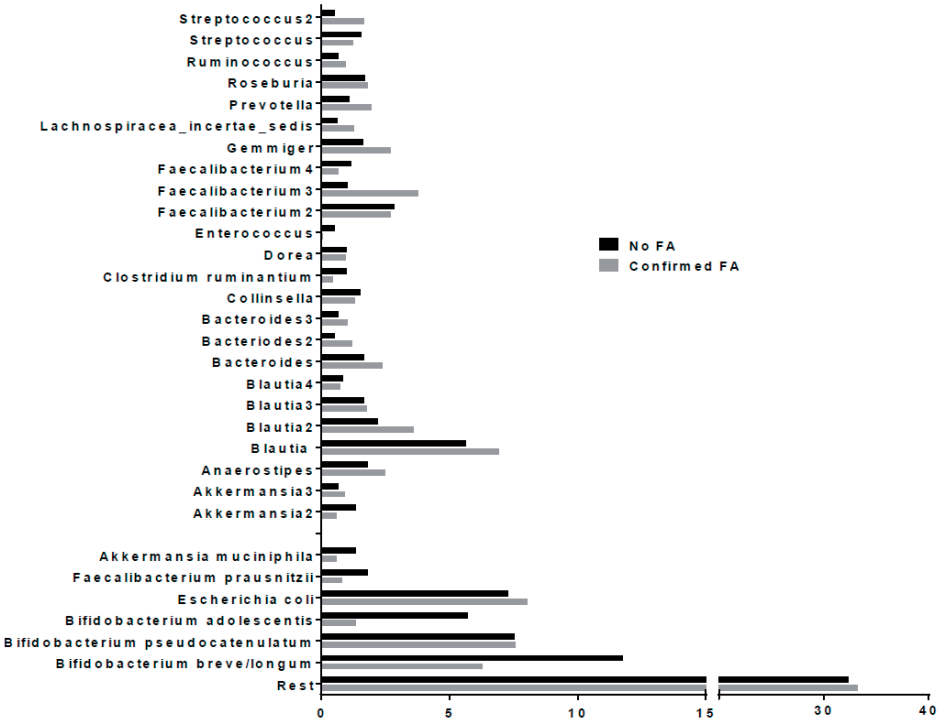

Figure S3. Distribution of six signature species in individual children with AD, without and with food allergy