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Fecal Microbiome and Food Allergy in Pediatric Atopic Dermatitis: A Cross-Sectional Pilot Study

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Keywords
Pediatric atopic dermatitis · Food allergy · Fecal microbiome · Intestinal microbiota · Microbes

Abstract
Background: Exposure to microbes may 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 atopic dermatitis (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 a 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 the presence and absence food allergy, were selected by elastic net regression. ***Results:*** Eighty-two children with AD (39 girls) with a median age of 2.5 years, and 20 of whom were diagnosed with food allergy, provided fecal samples. Food allergy to peanut and cow's milk was the most common. Six bacterial species from the fecal microbiome were identified, that, when combined, distinguished 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 the absence and presence of food allergy. Future studies are needed to confirm our findings.

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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 the intestinal microbiota and the subsequent development of atopic disease, including atopic dermatitis (AD), asthma, or rhinitis. However, few studies have investigated a specific link between the 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 modeling techniques are needed. Furthermore, the 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 hypothesized 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 the statistical elastic net regression approach.

Methods

Study Design and 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: a diagnosis of AD, an age of 0–18 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 compared 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 for 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 to take place 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 the level of specific IgE against common food allergens (hen's egg, cow's milk, peanut, hazelnut, fish, wheat, and soy). Both total and specific IgE were measured according to the manufacturer's protocol (Phadia, Uppsala, Sweden). A diagnosis of asthma or allergic rhinitis was based on the clinical history.

Food allergy was defined as a positive DBPCFC or a convincing clinical history, in combination with sensitization to a specific food, or, in the 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 h after the ingestion of the food. DBPCFC was considered positive and was then 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, and abdominal discomfort) on 3 subsequent doses, or the occurrence of a severe subjective symptom (abdominal pain/nausea with discomfort) lasting for >45 min, according to the international protocol [12]. Late reactions were assessed using follow-up by telephone the next day.

Fecal Samples

Fecal samples were collected at home and sent to the laboratory using the regular postal service. The samples were aliquoted and frozen at –20°C for further processing.

Fecal DNA Isolation

Approximately 150 mg of fecal material was directly transferred to the DNA isolation plate. Next, 0.5 mL phenol solution (pH 8.0; catalogue P4557, Sigma-Aldrich, St Louis, MO, USA) was added and the cells in the samples were mechanically disrupted by bead-beating twice for 3 min with a 96-well-plate BeadBeater (Biospec Products, Bartlesville, OK, USA). Samples were centrifuged at 4,000 rpm for 10 min 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 rDNA 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, USA). Barcoded DNA fragments spanning the archaeal and bacterial V4 hypervariable region were amplified with a standardizing level of template DNA (100 pg) to prevent overamplification. These amplicons, generated using adapted primers 533F and 806R, were bidirectionally sequenced using the MiSeq system [14]. Preprocessing and classification of sequences was performed using

Table 1. Patients' characteristics

	AD patients with no food allergy (n = 62)	AD patients with a confirmed food allergy (n = 20)	p value
Median age, years	3.0 (0.5–19)	2.2 (0.5–12)	0.606
Females, n	32 (52%)	7 (35%)	0.196
Median SA-EASI	29 (2–84)	46 (0–86)	0.283
Median TARC, pg/mL	1,243 (70–10,000)	2,251 (784–10,000)	0.013
Median total IgE, kU/L	62 (1.9–4,718)	564 (9–10,601)	<0.001
Sensitization to any food allergen, n	27 (43%)	20 (100%)	<0.001
On an 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

Values in parentheses after median values are min–max. AD, atopic dermatitis; FA, food allergy; SA-EASI, self-administered eczema area and severity index (scored by the research nurse); TARC, thymus and activation regulated chemokine.

modules implemented in the mothur v1.20.0 software platform [15]. The relative abundance of unique sequences was calculated for every fecal sample. The dataset was transformed using a zero mean unit variance transformation for subsequent statistical analyses. The V4 amplicon of the 16S rRNA encoding gene allows for the discrimination of several *Bifidobacteria* 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. Nonparametric tests were used to compare the groups with and without a confirmed food allergy.

Elastic Net Regression

Bacterial signature species discriminating between the 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, a tendency of shrinking coefficients to small values for correlated trending towards each other) [17, 18]. All species present 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 Receiver Operating Characteristics/Area under the Curve

A randomization test was conducted to test the statistical validity of the results obtained with elastic net regression. ROC/AUC (receiver operating characteristics/area under the curve) 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 v22 (IBM, Armonk, NY, USA) was used for descriptive data analysis. GraphPad Prism v6.01 (GraphPad Software, La Jolla, CA, USA) was used for providing graphs and figures. All other statistical analyses were performed using numerical Python v2.7 (Python Software Foundation, https://www.python.org).

Results

AD 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, and 20 children had a confirmed food allergy (Table 1). Of the 62 children without food allergy, almost half were sensitized to common food allergens without having symptoms of food allergy after ingestion of the food. In 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 2 children. On average, a DBPCFC was performed within 10 months (range 1–27 months) of providing the fecal samples.

Sequence and Microbiota Characteristics

A total of 2,609,478 high-quality sequences were obtained (mean 27,182; range 5,825–105,404 sequences/

Table 2. The number of children with confirmed food allergies

	A confirmed FA[a]	A positive DBPCFC[b]	An obvious clinical history (only)	FA was predicted, based on elevated sIgE to Ara h 2 (>5.17 kU/L)[c]
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%)			
Cashew			1 (5%)	
Pistachio			1 (5%)	
Soy	0			
Fish/shrimp	0			
Total	20 (100%)			

FA, food allergy. [a] Multiple FAs resulted in multiple entries: 1 patient had a confirmed FA to cow's milk, peanut, and hen's egg; 1 patient had a confirmed FA to hazelnut and hen's egg. [b] Including late reactions. [c] According to Klemans et al. [11].

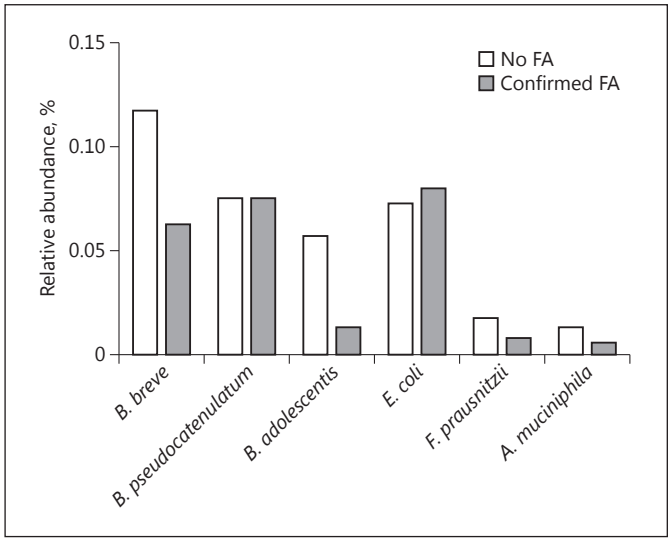

Fig. 1. Relative abundance of the microbial signature species in children with AD, without AD, and with a confirmed food allergy (FA).

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%), 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 6 microbial species from 4 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, *B. breve/longum* and *B. pseudocatenulatum/ catenulatum/ gallicum/ kashiwanohense* could not be distinguished after additional blasting in the RDP, and are referred to as *B. breve* and *B. pseudocatenulatum* throughout the paper.

Figure 1 shows the relative abundance of the 6 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* than children with AD without food allergy. The randomization test indicates that the combination of these 6 species is significantly different in the 2 groups ($p = 0.001$), even though the relative abundance of some single species may seem

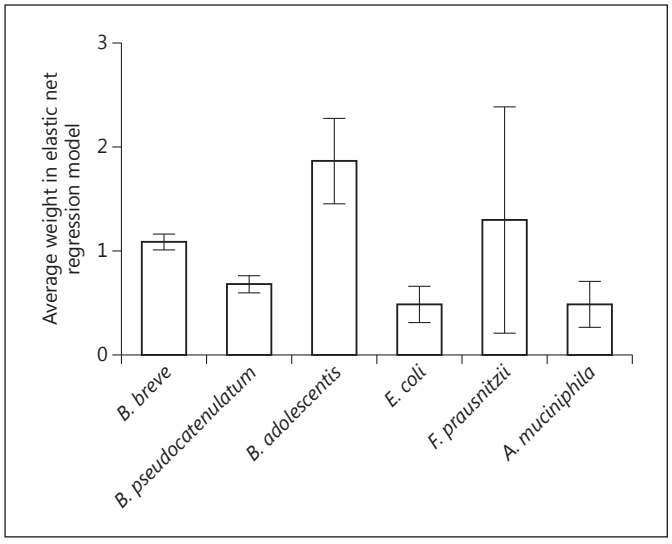

Fig. 2. Importance index for signature species in the elastic net regression model.

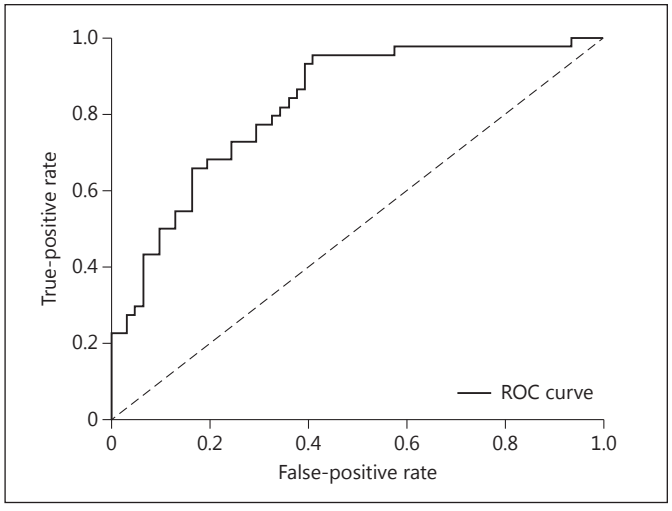

Fig. 3. Receiver operating characteristic (ROC) curve of the elastic net regression model. The area under the curve is 0.83.

similar on a group level (Fig. 1). Different relative contributions from the single species towards the total distinctive properties are distinguished, with *B. breve*, *B. adolescentis*, and *F. prausnitzii* having a greater influence than *B. pseudocatenulatum*, *E. coli*, and *A. muciniphila*, expressed as importance indices based on the elastic net regression (Fig. 2). The overall predictive accuracy of the classification model (AUC) is 0.83 (Fig. 3), with a sensitivity of 0.77 and a specificity of 0.80. Online supplementary Figures S1–S3 (for all online suppl. material, see www.karger.com/doi/10.1159/000484897) show the relative abundance of the signature species, the distribution of the 30 most abundant species, and the individual distribution of the signature species.

Discussion

We analyzed the fecal microbiome of children with AD with or without a concomitant food allergy, and found that a combination of 6 microbial species, including *E. coli*, *F. prausnitzii*, *A. muciniphila*, and 3 types of *Bifidobacteria*, discriminates between the presence and absence of food allergy in children with AD ($p = 0.001$). 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* than that of children with AD without food allergy. We found no differences in microbial diversity (according to the Shannon index) between the children 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 by making use of 16S rRNA sequencing techniques to generate 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 a 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 than with other statistical regression approaches [22].

Our study demonstrates that children with AD and a food allergy had significantly less *F. prausnitzii* and *A. muciniphila* than 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 common 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 pro-

ducer 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 the 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 *B. breve* and *B. adolescentis* were mainly contributed by the children allergic to cow's milk. Furthermore, we found an increased relative abundance of *E. coli* in the food-allergic group. *E. coli* has previously been associated with the 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 levels of thymus and activation regulated chemokine (TARC) in the food-allergic group suggest increased AD severity compared to the nonallergic group. This raises the possibility that the selected biomarkers also correlated with AD severity, which fits with the observation that the prevalence of food allergy is higher in children with greater disease severity [32].

All the microbial species resulting from our analysis have previously been correlated with atopic disease in other studies. This might raise the question of 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 were confirmed or ruled out based on the child's clinical history. Food allergy was diagnosed based on DCPCFC in the majority of patients. Post hoc analyses showed no significant differences between the group with and the group without food allergy with regard to other atopic diagnoses, suggesting that the species identified species indicate food allergy rather than general atopy.

Our study supports the hypothesis that, in children with AD, the intestinal microbiome differs in children with and without food allergy. Intestinal microbiota regulate the development of functions of a diverse range of T cells, such as Th17, Th1, Th2, and regulatory T cells, and also modulate innate lymphoid cells [33, 34]. By modifying the response of gut-associated lymphoid tissues, intestinal microbiota may influence the development of oral tolerance [35]. A recent study on 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 epithelial integrity of the gut, thereby increasing the risk of allergic sensitization through the direct uptake of allergens [7]. However, the exact mechanisms by which the intestinal microbiome influences food allergy are not elucidated yet. Furthermore, it is not clear whether a change in the 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 to this, established food allergies lead to an elimination diet, where the specific food allergen is excluded from the general diet. We cannot exclude the possibility that an elimination diet where one particular food is excluded from the diet would also leads to detectable changes in the fecal microbial composition, as has been demonstrated where there is an increased consumption of specific foods [38]. However, in our study, one-third of the children in the group without food allergy also reported being on an elimination diet for a specific food, for various reasons. Furthermore, dietary intake varies according to personal preference. Therefore, it is unlikely that the observed microbial differences can be solely attributed to the dietary differences. Besides a self-reported elimination diet, dietary intake was not further assessed in this study because it is very difficult to assess accurately.

A limitation of our cross-sectional study was the heterogeneity of the study population. All of the children were diagnosed with AD, with varying disease severity, age, and food allergies. We did not include any healthy controls. As was to be 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 were influenced by the contribution of these particular 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 the

use of antibiotics, birth via caesarean section, or breastfeeding, 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 allergy 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, preferably with prospective study designs using well-defined patient populations to further explore the potential of the fecal microbial colonization patterns associated with specific food allergies in children with AD. Control groups should also be included, i.e., children with food allergy without AD, or with severe "extrinsic" AD without food allergy. These groups should be of sufficient size to allow for the stratification of different food allergies.

Conclusion

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

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Disclosure Statement

The authors have no conflicts of interest to declare.

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