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A prospective microbiome-wide association study of food sensitization and food allergy in early childhood

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

Abstract

Background—Alterations in the intestinal microbiome are prospectively associated with the development of asthma; less is known regarding the role of microbiome alterations in food allergy development.

Methods—Intestinal microbiome samples were collected at age 3–6 months in children participating in the follow-up phase of an interventional trial of high-dose vitamin D given during pregnancy. At age 3, sensitization to foods (milk, egg, peanut, soy, wheat, walnut) was assessed. Food allergy was defined as caretaker report of healthcare provider-diagnosed allergy to the above foods prior to age 3 with evidence of IgE sensitization. Analysis was performed using Phyloseq and DESeq2; *P*-values were adjusted for multiple comparisons.

Results—Complete data were available for 225 children; there were 87 cases of food sensitization and 14 cases of food allergy. Microbial diversity measures did not differ between food sensitization and food allergy cases and controls. The genera *Haemophilus* ($\log_2$ fold change −2.15, P=.003), *Dialister* ($\log_2$ fold change −2.22, P=.009), *Dorea* ($\log_2$ fold change −1.65, P=.02), and *Clostridium* ($\log_2$ fold change −1.47, P=.002) were underrepresented among subjects with food sensitization. The genera *Citrobacter* ($\log_2$ fold change −3.41, P=.03), *Oscillospira* ($\log_2$ fold change −2.80, P=.03), *Lactococcus* ($\log_2$ fold change −3.19, P=.05), and *Dorea* ($\log_2$ fold change −3.00, P=.05) were underrepresented among subjects with food allergy.

Conclusions—The temporal association between bacterial colonization and food sensitization and allergy suggests that the microbiome may have a causal role in the development of food allergy. Our findings have therapeutic implications for the prevention and treatment of food allergy.

Keywords

Dorea; food allergy; food sensitization; microbiome

1 | INTRODUCTION

Food allergies cause life-threatening anaphylactic reactions in affected children and adults and lead to significant morbidity, quality of life impairment, and healthcare costs.[1,2] The prevalence of food allergy has markedly increased over a recent time period.[3] Although the cause of food allergy is not currently known, it is generally accepted that changes in microbial exposures in line with the hygiene hypothesis of allergic disease, or the “gut microbial deprivation hypothesis,” may in part explain the recent rise in prevalence of food allergy.[4–7]

To date, several studies have investigated the relationship between the microbiome and development of allergic disease. Both diversity of microbial exposures and specific taxa are thought to be protective against development of allergy and asthma.[8] The study of the microbiome and food allergy specifically has been less fruitful, in part because of the lower prevalence of food allergy, and challenges with accurate phenotyping of this disease. In a small prospective study, richness (a measure of microbial diversity) of the intestinal microbiota was inversely associated with the development of food sensitization, while the

Enterobacteriaceae/Bacteroidaceae ratio was positively associated with the development of food sensitization at age 1 year.[9] Animal models, which can elucidate disease mechanisms, highlight a protective role for members of the class Clostridia in food sensitization via induction of IL-22 production by innate lymphoid cells with subsequent reduction of allergen exposure to the circulation.[10] Clostridia were also enriched in the stool of infants with milk allergy who outgrew their allergy by age 8 years, compared to those whose allergy did not resolve by that time.[11]

We performed a microbiome-wide association study of the infant intestinal flora and the development of food sensitization and food allergy in a prospective birth cohort of 216 children. Our objective was to determine whether gut microbial composition was associated with food sensitization and allergy and whether these associations might be along the causal pathway for food allergy or explained by other environmental exposures. To our knowledge, this is the largest prospective study of the microbiome and development of food sensitization and allergy performed to date.

2 | METHODS

2.1 | Source of subjects

The Vitamin D Antenatal Asthma Reduction Trial (VDAART) is a randomized, controlled trial of high (4000IU)- vs standard-dose (400IU) daily vitamin D supplementation during pregnancy. The study design and primary outcome have been published previously.[12,13] Briefly, VDAART enrolled 881 pregnant women at weeks 10–18 of pregnancy at three clinical sites in the United States (Boston, MA; St. Louis, MO; and San Diego, CA). Inclusion criteria included a personal history of asthma or allergy in the pregnant woman or in the father of the fetus. Smoking, other chronic disease, multiple gestation pregnancy, and assisted reproduction were exclusion criteria. The intervention phase occurred during pregnancy, and after birth, infants were followed every 3 months for the development of respiratory disease and other outcomes, including diagnosis of food allergy by a healthcare provider.

An ancillary study of the child intestinal microbiome was initiated during follow-up, after roughly half of the children reached 3 months of age. Stool samples were collected from infants between ages 3 and 6 months. Caretakers were instructed to collect ½ teaspoon of child stool from a diaper using a tongue depressor 1–2 days prior to a study visit and to store the sample in the freezer at home until bringing it to the research clinic in a freezer pack. Antibiotic use by the infant in the past 7 days was an exclusion criterion for stool collection.

2.2 | Variable definitions

At age 3 years, laboratory testing for serum-specific IgE to milk, egg, peanut, soy, wheat, and walnut was performed (Thermo Scientific/Phadia Immunology Reference Laboratory, Uppsala, Sweden). A child was considered to have food sensitization if any food-specific IgE was greater than 0.35 kU/L. Two children had missing data for walnut IgE and had sensitization results for the other foods. Neither child reported a history of walnut allergy and were considered nonsensitized to walnut.

Telephone or in-person questionnaires were performed every 3 months following birth. Caretakers were asked “since [birth/the last time we talked], has a health care provider said that [CHILD] has food allergies?” If the answer was affirmative, the caretaker was asked “which foods did the health care provider say [CHILD] is allergic to?” Possible responses included milk, egg, peanut, wheat, soy, other nuts, shellfish, fish, with the option to report other food allergies as free text. A child was considered to have food allergy if there was caretaker report of healthcare provider-diagnosed allergy to milk, egg, peanut, wheat, soy, or other nut allergy prior to age 3 years with evidence of IgE sensitization to that food (>0.1 kU/L) at age 3 years.

Other study variables included self-reported race and ethnicity of the child (categorized as African American, Hispanic, White, and Other), maternal vitamin D supplementation treatment assignment, child gender, mode of delivery (vaginal vs C-section), age of child at stool collection, and whether the child was ingesting formula and/or breast milk and solid food at the time of the stool collection. Per study protocol, questions regarding the child diet were asked beginning at age 6 months. Because the stool collection occurred between the ages of 3 and 6 months, we used questionnaire responses obtained at age 6 months (or later if 6-month data were incomplete) to determine whether the child was on breast milk, infant formula, or solid food at the time of stool collection. For additional details on dietary variables, please see the Appendix S1. Subjects were included in this study if infant gut microbial characterization and sensitization data were available. Missing data were considered missing at random.

2.3 | Stool sample sequencing and taxonomic identification

Sequencing of the bacterial 16S V3 to V5 hypervariable regions was performed using the Roche 454 Titanium platform. Filtering, trimming, and chimera checking were performed as previously described.[14,15] We used closed-reference operational taxonomic unit (OTU) classification in Qiime to group sequences according to sequence similarity and match to taxonomy.[16] We excluded samples with total read counts <1000 and OTUs identified less than 10 times or in fewer than 10 subjects.

2.4 | Statistical analysis

Distributions of potential confounders were compared by food sensitization or food allergy status using the chi-square test. We calculated richness (Chao1) and diversity (Shannon index) at the genus level and compared these between sensitization and allergy groups using the *t* test.

We performed a microbiome-wide analysis to identify bacterial genera that were differentially abundant between cases and controls. We used a negative binomial model without rarefying to account for variability in read depth between samples following the recommendations of McMurdie and Holmes.[17] Reported *P*-values were corrected for multiple comparisons using the method of Benjamini and Hochberg.[18] Analyses were performed in Stata 12 (College Station, TX, USA) and the Phyloseq and DESeq2 packages of R.[19,20] We stratified our analyses by potential confounders in multiple sensitivity analyses.

3 | RESULTS

A total of 810 children were born to VDAART participants who were randomized to treatment. The 216 subjects in this study included those with data on food sensitization and infant gut microbial characteristics, and were similar demographically to subjects who did not have these data available (data not shown). Complete phenotypic data were available on all study subjects with the exception of missing data for breastfeeding (n=3) and solid food introduction (n=2) at the time of stool collection (Table 1).

There were 85 subjects with food sensitization and 131 subjects without food sensitization. The majority (79%) of food-sensitized subjects were sensitized to milk, with egg being the next most common sensitization with 56% sensitized. Food-sensitized subjects tended to be African American (55%) while nonsensitized subjects were more evenly distributed across race and ethnicity (35% African American, 34% Hispanic, 25% White; P=.02). A greater percentage of food-sensitized subjects had been introduced to solid food by the time of stool collection compared to nonsensitized subjects (54% vs 41%, P=.05).

There were 14 subjects with food allergy. These subjects reported a history of healthcare provider-diagnosed milk, egg, peanut, wheat, soy, or other nut allergy with onset between the ages of 9 months and 3 years and had evidence of sensitization to those foods at age 3 years. Egg and peanut allergy were the most common food allergies, reported in nine and eight subjects, respectively, while allergy to the other foods occurred in two to three subjects each. Subjects with food allergy were in general similar to subjects without food allergy, although more subjects with food allergy had a history of C-section delivery (57% vs 30%, P=.04).

3.1 | Microbiome diversity, food sensitization, and allergy

Measures of intestinal diversity were similar between subjects with and without food sensitization (mean Chao1 diversity index 31 vs 31, P=.26; mean Shannon diversity index 2.13 vs 2.21; P=.11) and with and without food allergy (mean Chao1 diversity index 32 vs 31, P=.67; mean Shannon diversity index 2.19 vs 2.17; P=.54; Table 2).

3.2 | Microbiome-wide analysis of food sensitization and allergy

Prominent bacterial families across all groups included Bacteroidaceae, Enterobacteriaceae, and Lachnospiraceae (Figure 1). Microbiome-wide analysis was performed at the genus taxonomic rank. Four genera were statistically significantly associated with food sensitization; all had lower read counts in cases compared to controls: *Haemophilus* ($\log_2$ fold change −2.15, P_{BH}=.003), *Dialister* ($\log_2$ fold change −2.22, P_{BH}=.009), *Dorea* ($\log_2$ fold change −1.65, P_{BH}=.02), and *Clostridium* ($\log_2$ fold change −1.47, P_{BH}=.02; Figure 2, Table 3). No genera had statistically significantly increased relative abundance in cases compared to controls; there was a trend toward higher read counts for *Dysgonomonas* in those with food sensitization compared to controls (log2 fold change 2.26, P_{BH}=.29).

Four genera were statistically significantly associated with food allergy; again, all had lower read counts in cases compared to controls: *Citrobacter* ($\log_2$ fold change −3.41, P_{BH}=.03), *Oscillospira* ($\log_2$ fold change −2.80, P_{BH}=.03), *Lactococcus* ($\log_2$ fold change −3.19, P_{BH}=.

05), and *Dorea* ($\log_2$ fold change −3.00, P_{BH}=.05; Figure 2, Table 4). No genera had statistically significantly increased relative abundance in cases compared to controls; there was a trend toward higher read counts for SMB53 (candidate genus of the family Clostridiaceae) and *Stenotrophomonas* in those with food allergy compared to controls (log2 fold change 2.29 and 2.20, respectively, and P_{BH}=.05 and .37, respectively).

3.3 | Sensitivity analyses

Because methods to perform an adjusted microbiome-wide association study in the prediction of food allergy are to our knowledge currently limited, we repeated our initial analyses stratified by potential confounders of the relationship between the microbiome and food sensitization and allergy that were identified in Table 1: race/ethnicity and solid food feeding for food sensitization and C-section history for food allergy. We found in general that among subgroups of subjects, the direction and magnitude of point estimates for the association between *Haemophilus*, *Dialister*, *Dorea*, and *Clostridium* and food sensitization, and between *Citrobacter*, *Oscillospira*, *Lactococcus*, and *Dorea* and food allergy were similar to those identified in the larger cohort (Tables 5–6). However, the association between food sensitization and *Clostridium* was reduced in magnitude and changed direction in the African American subgroup as well as the subgroup that had been introduced to solid food. The associations between food allergy and *Citrobacter, Oscillospira, Lactococcus, and Dorea* persisted after stratification by mode of delivery.

4 | DISCUSSION

We conducted a microbiome-wide association study of the infant gut microbiome and food sensitization and food allergy in early childhood. After correction for multiple comparisons, we identified four bacterial genera present in the infant intestinal microbiome that were significantly inversely associated with the development of food sensitization at the age of 3 years and four genera that were significantly inversely associated with the development of food allergy by age 3 years. Notably, the genus *Dorea* was associated with both food sensitization and food allergy. We completed a sensitivity analysis indicating that our results are generally robust to potential confounders, although associations between *Clostridia* and food sensitization may be confounded by race and diet.

We identified seven individual genera with significant inverse associations with food sensitization and/or allergy. Two members of the phylum Proteobacteria were inversely associated with food sensitization (*Haemophilus*) or allergy (*Citrobacter*). These gram-negative bacteria are better known for their role in disease than in health, although commensal members are known to exist. Additionally, prior studies investigating the association of the intestinal microbiome during infancy and allergic disease in humans have demonstrated similar inverse associations between *Haemophilus* and eczema,[21] and *Citrobacter* and allergic sensitization and asthma.[22]

Five members of the phylum Firmicutes, predominantly gram-positive bacteria which make up a majority of the intestinal microbiome, were inversely associated with food sensitization or allergy.[23] The Firmicutes include the class Clostridia, which has recently been identified as protective against peanut sensitization in a mouse model of food allergy.[10] Clostridia may

also speed the resolution of milk allergy.[11] The class Clostridia includes the genera *Dorea*, which was significantly reduced among children with food sensitization and allergy in the current study; *Clostridium* reduced in children with food sensitization, and *Oscillospira* reduced among children with food allergy. The Clostridia are widely appreciated for their ability to cause disease in *Clostridium difficile* infection, and indeed the presence of *C. difficile* in the infant microbiome has been positively associated with atopic outcomes in later childhood.[24–26] However, Clostridia class members are also increasingly recognized as important in health promotion.[27] Many have anti-inflammatory properties through their ability to produce short-chain fatty acids[28,29] or via other mechanisms leading to reduced IL-12 and IFN-γ levels and secretion of IL-10.[30] *Dialister* was formerly recognized as a member of the class Clostridia, as it is closely related to this class. *Lactococcus*, of the class Bacilli, is the only non-Clostridial Firmicute associated with food allergy in the current study and has anti-inflammatory properties such as reduction of TNF-α.[31] Similar relationships between the Firmicutes identified in our analysis and other allergic disorders have been seen in prior studies of the human intestinal microbiome during infancy: *Dorea* has been inversely associated with both eczema and allergic sensitization and asthma,[21,22] *Dialister* has been inversely associated with eczema,[21] and *Lactococcus*, *Clostridium*, and *Oscillospira* have been inversely associated with atopy and asthma.[22,32] While the Firmicutes mentioned above do have biological activity suggesting that a potential role in susceptibility to food sensitization or allergy is plausible, further work is necessary in humans and animal models to understand the strength and mechanism of these associations.

Although we did identify individual genera that were significantly associated with food sensitization and allergy, global microbiome measures of diversity were similar between cases and controls. This was surprising in light of several reports of inverse associations between intestinal microbial diversity and risk of allergic diseases including eczema[33] and asthma[34–36] using methods similar to ours. The one study that reported an inverse association between infant intestinal microbial diversity at age 3 months and food sensitization at age 1 year used family taxonomic rank measurements and included only 12 cases of food sensitization, factors that may in part underlie the discrepancy in findings.[9]

The strengths of our study include our large and diverse study population, prospective study design, and analysis of the entire metagenome using sequencing-based methods. Although the number of food allergy cases (defined by history and detectible IgE at age 3 years) was low, this is similar to what has been published in case-control studies without the benefit of our prospective design.[37]

Our study has several limitations. Unlike traditional genomewide association studies, the methodology for analyzing the microbiome as a predictor of disease while accounting for potential confounding by other exposures has not, to our knowledge, been standardized. We therefore evaluated the effect of each known potential confounder independently of other confounders using stratified analyses, as we were methodologically unable to complete a true multivariate association study. We found that our overall findings were relatively robust to confounding, except perhaps to race; however, we may be limited by unmeasured confounders that we could not address with our dataset. Further, we chose to analyze the microbiome at the genus level as we did not have the power to identify meaningful

associations when over 1000 individual taxa (species or OTUs) were included in the analysis. Therefore, we are limited in the ability to fully resolve the identity of taxa that may be important to the development of food allergy. Finally, although we are reasonably confident in our case definitions of food sensitization and food allergy, some children who did develop and then resolve food allergy may have been misclassified as controls, which may have biased our results toward the null.

In summary, we identified a bacterial genus, *Dorea*, which is reduced in the intestinal microbiomes of infants who later develop food sensitization and food allergy. Our results suggest that *Dorea* may promote or protect against food sensitization and food allergy. Further work to identify the specific relevant species and strains may lead to prevention or treatment of food allergy through altering the developing infant microbiome.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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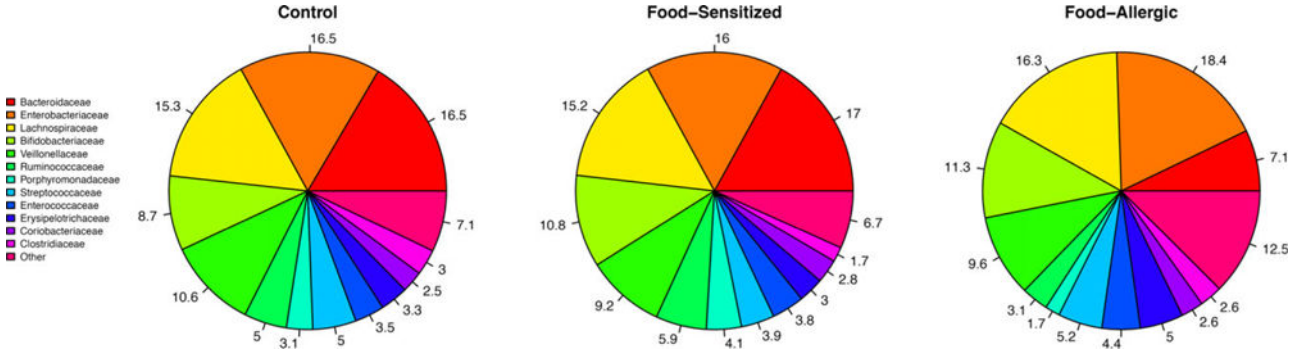

FIGURE 1.

Bacterial relative abundances at the family taxonomic rank by food allergy or sensitization status

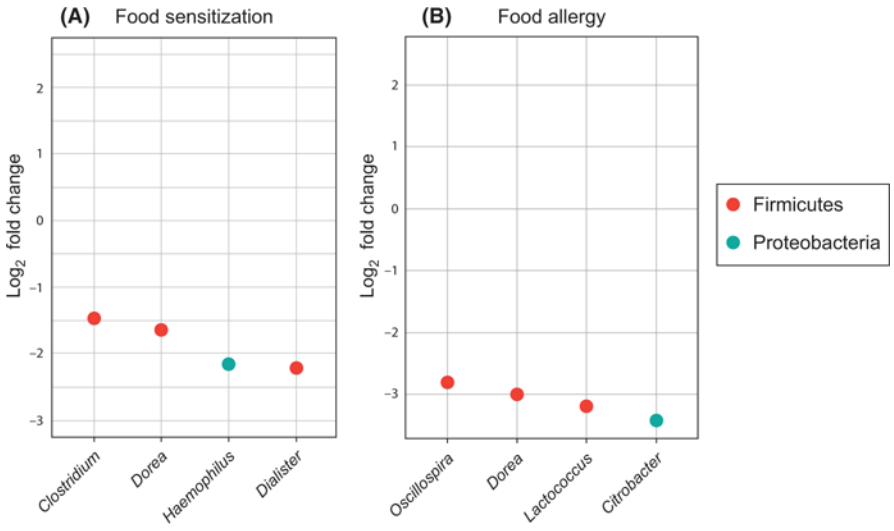

FIGURE 2.

Significant associations between food sensitization A, and food allergy B, and bacterial genera. The $\log_2$ fold change is plotted for each bacterial genus significantly associated with food sensitization or food allergy

TABLE 1

Demographics of 216 children providing evaluable stool samples for food sensitization and food allergy analysis

	Food sensitization N=85	No food sensitization N=131	*P*	Food allergy N=14	No food allergy N=202	*P*
Milk	67 (79)			3 (21)		
Egg	48 (56)			9 (64)		
Peanut	28 (33)			8 (57)		
Soy	15 (18)			2 (14)		
Wheat	33 (39)			3 (21)		
Walnut	11 (13)			2 (14)		
Race						
African American	47 (55)	46 (35)	.02	7 (50)	86 (43)	.67
Hispanic	16 (19)	45 (34)		3 (21)	58 (29)	
White	16 (19)	33 (25)		4 (29)	45 (22)	
Other	6 (7)	7 (5)		0	13 (6)	
Vitamin D Treatment[a]	48 (57)	77 (59)	.74	8 (57)	117 (58)	.96
Female	38 (45)	65 (50)	.48	8 (57)	98 (47)	.46
C-section	25 (29)	44 (34)	.52	8 (57)	61 (30)	.04
Formula Fed	63 (74)	84 (64)	.12	9 (64)	138 (68)	.75
Breast fed	32 (38)	56 (44)	.38	7 (50)	81 (41)	.50
Solid food	45 (54)	53 (41)	.05	8 (57)	90 (45)	.38
Age at infant stool collection (mean, range)	148 (92–208)	140 (78–206)	.95	153 (115–208)	142 (78–206)	.87
Read counts of microbial sample (mean, range)	5282 (1726–12 655)	5310 (1236–19 917)	.48	5351 (2281–9387)	5295 (1236–19 917)	.53

[a]High-dose vitamin D supplementation during pregnancy, as part of the Vitamin D Antenatal Asthma Reduction Trial study.

TABLE 2

Intestinal microbial diversity and food sensitization and allergy

Outcome	Diversity measure	Cases	Controls	*P*-value
Food sensitization	Chao1	31 (13–55)	31 (9–52)	.26
	Shannon	2.13 (0.70–3.15)	2.21 (0.50–2.99)	.11
Food allergy	Chao1	32 (23–44)	31 (9–55)	.67
	Shannon	2.19 (1.51–2.65)	2.17 (0.50–3.15)	.54

Values presented are mean (range).

TABLE 3

Significant associations between microbial genera and food sensitization

Rank	Phylum	Family	Genus	Base mean	log2 Fold Change	P_{adj}
1	Proteobacteria	Pasteurellaceae	*Haemophilus*	12.52	−2.15	.003
2	Firmicutes	Veillonellaceae	*Dialister*	2.04	−2.22	.009
3	Firmicutes	Lachnospiraceae	*Dorea*	22.9	−1.65	.02
4	Firmicutes	Clostridiaceae	*Clostridium*	32.6	−1.47	.02

TABLE 4

Significant associations between microbial genera and food allergy

Rank	Phylum	Family	Genus	Base mean	log2 Fold Change	P_{adj}
1	Proteobacteria	Enterobacteriaceae	*Citrobacter*	6.14	−3.41	.03
2	Firmicutes	Ruminococcaceae	*Oscillospira*	23.2	−2.80	.03
3	Firmicutes	Streptococcaceae	*Lactococcus*	8.3	−3.19	.05
4	Firmicutes	Lachnospiraceae	*Dorea*	22.8	−3.00	.05

TABLE 5

Sensitivity analyses for food sensitization

Subset	n	Rank	Phylum	Family	Genus	Base mean	log2 Fold Change	P_{adj}
African American	93	65	Proteobacteria	Pasteurellaceae	*Haemophilus*	4.29	−0.34	.99
		5	Firmicutes	Veillonellaceae	*Dialister*	2.71	−1.81	.33
		6	Firmicutes	Lachnospiraceae	*Dorea*	23.60	−1.22	.62
		56	Firmicutes	Clostridiaceae	*Clostridium*	8.52	0.42	.99
Non-African American	123	2	Proteobacteria	Pasteurellaceae	*Haemophilus*	16.10	−2.37	.02
		4	Firmicutes	Veillonellaceae	*Dialister*	1.03	−2.39	.12
		6	Firmicutes	Lachnospiraceae	*Dorea*	22.00	−1.95	.16
		7	Firmicutes	Clostridiaceae	*Clostridium*	45.88	−1.49	.19
Solid Food	98	11	Proteobacteria	Pasteurellaceae	*Haemophilus*	6.97	−1.40	.41
		26	Firmicutes	Veillonellaceae	*Dialister*	0.94	−0.94	.94
		12	Firmicutes	Lachnospiraceae	*Dorea*	21.04	−1.17	.42
		27	Firmicutes	Clostridiaceae	*Clostridium*	8.41	0.58	.94
No solid food	118	1	Proteobacteria	Pasteurellaceae	*Haemophilus*	14.68	−2.33	.15
		3	Firmicutes	Veillonellaceae	*Dialister*	3.00	−2.35	.17
		4	Firmicutes	Lachnospiraceae	*Dorea*	24.91	−2.06	.17
		5	Firmicutes	Clostridiaceae	*Clostridium*	39.45	−1.54	.27

TABLE 6

Sensitivity analyses for food allergy

Subset	n	Rank	Phylum	Family	Genus	Base mean	log2 Fold Change	P_{adj}
C-section	69	1	Proteobacteria	Enterobacteriaceae	*Citrobacter*	6.23	−4.15	.09
		4	Firmicutes	Ruminococcaceae	*Oscillospira*	27.15	−2.25	.39
		6	Firmicutes	Streptococcaceae	*Lactococcus*	7.47	−2.81	.43
		13	Firmicutes	Lachnospiraceae	*Dorea*	35.88	−1.82	.75
No C-section	147	44	Proteobacteria	Enterobacteriaceae	*Citrobacter*	4.92	−2.00	.99
		3	Firmicutes	Ruminococcaceae	*Oscillospira*	18.33	−3.95	.03
		8	Firmicutes	Streptococcaceae	*Lactococcus*	8.36	−2.53	.99
		2	Firmicutes	Lachnospiraceae	*Dorea*	7.93	−4.68	.03