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ORIGINAL ARTICLE **Clinical Mechanisms in Allergic Disease**

Infant gut microbiota and food sensitization: associations in the first year of life

M. B. Azad[1,2], T. Konya[3], D. S. Guttman[4], C. J. Field[5], M. R. Sears[6], K. T. HayGlass[7], P. J. Mandhane[1], S. E. Turvey[8], P. Subbarao[9], A. B. Becker[2], J. A. Scott[3] and A. L. Kozyrskyj[1,10] and the CHILD Study Investigators*

[1]Department of Pediatrics, School of Public Health, University of Alberta, Edmonton AB, [2]Department of Pediatrics & Child Health, Children's Hospital Research Institute of Manitoba, University of Manitoba, Winnipeg, MB, [3]Dalla Lana School of Public Health, University of Toronto, [4]Centre for the Analysis of Genome Evolution and Function, University of Toronto, Toronto, ON, [5]Department of Agricultural, Food & Nutritional Science, University of Alberta, Edmonton, AB, [6]Department of Medicine, McMaster University, Hamilton, ON, [7]Department of Immunology, University of Manitoba, Winnipeg MB, [8]Department of Pediatrics, Child & Family Research Institute, BC Children's Hospital, University of British Columbia, Vancouver, BC, [9]Department of Pediatrics, Hospital for Sick Children, University of Toronto, Toronto ON, and [10]Department of Community Health Sciences, University of Manitoba, Winnipeg, MB, Canada

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Correspondence:
Prof. Anita Kozyrskyj, Department of Pediatrics, University of Alberta, 3-527 Edmonton Clinic Health Academy, 11405–87th Avenue, Edmonton, AB, Canada T6G IC9.
E-mail: kozyrsky@ualberta.ca
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Summary

Background The gut microbiota is established during infancy and plays a fundamental role in shaping host immunity. Colonization patterns may influence the development of atopic disease, but existing evidence is limited and conflicting.

Objective To explore associations of infant gut microbiota and food sensitization.

Methods Food sensitization at 1 year was determined by skin prick testing in 166 infants from the population-based Canadian Healthy Infant Longitudinal Development (CHILD) study. Faecal samples were collected at 3 and 12 months, and microbiota was characterized by Illumina 16S rRNA sequencing.

Results Twelve infants (7.2%) were sensitized to ≥ 1 common food allergen at 1 year. Enterobacteriaceae were overrepresented and Bacteroidaceae were underrepresented in the gut microbiota of food-sensitized infants at 3 months and 1 year, whereas lower microbiota richness was evident only at 3 months. Each quartile increase in richness at 3 months was associated with a 55% reduction in risk for food sensitization by 1 year (adjusted odds ratio 0.45, 95% confidence interval 0.23–0.87). Independently, each quartile increase in Enterobacteriaceae/Bacteroidaceae ratio was associated with a twofold increase in risk (2.02, 1.07–3.80). These associations were upheld in a sensitivity analysis among infants who were vaginally delivered, exclusively breastfed and unexposed to antibiotics. At 1 year, the Enterobacteriaceae/Bacteroidaceae ratio remained elevated among sensitized infants, who also tended to have decreased abundance of Ruminococcaceae.

Conclusions and Clinical Relevance Low gut microbiota richness and an elevated Enterobacteriaceae/Bacteroidaceae ratio in early infancy are associated with subsequent food sensitization, suggesting that early gut colonization may contribute to the development of atopic disease, including food allergy.

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Introduction

Sensitization to food allergens is common during the preschool years, affecting up to 28% of children in the US [1]. While the majority of food-sensitized infants will not develop food allergy [2, 3], they are more likely to experience the 'atopic march' to conditions such as atopic dermatitis, allergic rhinitis and asthma [4–10]. Further, food sensitization has been identified as a first indication for failure of allergen avoidance measures to prevent future atopic disease [8, 11].

Drawing on the hygiene hypothesis, early life environmental exposures that modify infant contact with microbes are being investigated to better understand what predisposes some children to develop food allergy

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while others do not. This search has yielded several tentative risk factors during the perinatal period, such as caesarean section delivery, early cessation of breastfeeding and antibiotic treatment. Summarized in a recent review by Molloy et al. [12], there is inconsistent evidence for an association between caesarean delivery and proven food allergy. Some studies report increased risk of food allergy among children born by caesarean section, particularly when the mother is atopic [13, 14], but others find no evidence of association [15–17]. More consistently documented, however, is that sensitization to milk and egg allergens is twice as likely to occur in children born by caesarean section [18, 19]. Exposure to antibiotics during the neonatal period has been shown to enhance food sensitization in mice [20], but findings from human studies are conflicting [12], although incomplete maternal recall of antibiotic use may contribute to this inconsistency. Lastly, despite much research, the role of breastfeeding in preventing food allergy remains uncertain [12], and evidence for other putative protective factors such as having siblings or attending day care is limited [21].

Despite evidence that perinatal risk factors for food allergy can also modify the infant gut microbiota [22–24], direct evidence of microbiota disruption in atopic individuals is limited, particularly with respect to food sensitization. Lower gut microbiota diversity and relative abundance of *Bacteroides* by 1 month of age have been reported in infants subsequently diagnosed with atopic dermatitis [25, 26]. Low diversity during infancy has also been associated with increased risk of allergic sensitization at school age [27]. In a Dutch birth cohort study, atopic sensitization at 2 years of age was predicted by greater colonization with *Clostridium difficile* at 1 month [28]. At 18 months of age, Nylund et al. [29] also observed lower *Bacteroides* and higher relative abundance of *Clostridium* clusters among children subsequently diagnosed with atopic dermatitis. While food allergens were among those tested in these studies, neither food allergy nor sensitization was reported as a separate outcome. Specific to milk allergy at 6 months of age, Thompson-Chagoyan et al. [30] documented greater counts of cultured anaerobes from infants with confirmed allergy to cow's milk, as well as more frequent colonization with *Clostridium coccoides* [31], when compared to controls. Most recently, Ling et al. [32] reported higher Firmicutes and lower Bacteroidetes abundance among 5-month-old Chinese infants with confirmed food allergy, with no difference in overall microbiota diversity. As both of these food allergy studies were cross-sectional, causality could not be inferred.

Against this backdrop of conflicting evidence for the role of perinatal events in the development of food allergy and a scarcity of studies evaluating associations between early gut microbiota dysbiosis and food allergy or sensitization, we aimed to determine whether food sensitization at 1 year of age was associated with prior or concurrent gut microbiota composition and diversity. Our second objective was to determine whether these associations existed in the absence of major established early life risk factors for microbiota dysbiosis.

Methods

Study design and covariates

This study of 166 infants represents a subset of the larger Canadian Healthy Infant Longitudinal Development (CHILD) national population-based birth cohort (www.canadianchildstudy.ca). Participants were enrolled in Winnipeg, Manitoba, Canada between June 2009 and January 2011. Microbiome analyses were conducted for an unselected subsample comprising the first 166 enrolled infants with available faecal samples at 3 months and 1 year of age, and complete allergy skin prick testing results at 1 year. Table S1 shows demographic characteristics of the full Winnipeg CHILD cohort compared to the subsample assessed here, showing no major differences. Mothers completed standardized questionnaires at 3, 6, and 12 months postpartum, reporting on potential risk factors for food sensitization [33], including: infant food allergy symptoms, rash, medications, diet (breastfeeding duration and exclusivity, timing of solid food introduction), household pets, siblings and maternal food allergy (positive response to the question 'Have you ever had a food allergy?'). Mode of delivery and hospital antibiotic exposures were obtained from hospital records. Infant antibiotic exposure was classified at 3 and 12 months as indirect only (mother received intrapartum antibiotics, but infant never received antibiotics directly), direct (parent-reported oral prescription or documented administration of antibiotics in hospital; with or without indirect exposure) or no exposure. Atopic dermatitis was diagnosed at the 1-year clinical assessment according to British Association of Dermatologists criteria [34]. Written informed consent was obtained from parents at enrolment. This study was approved by the University of Manitoba Human Research Ethics Board.

Sample collection, DNA extraction and amplification

Faecal samples (fresh or refrigerated for a short period) were collected at a home visit (3 months) or brought to a clinic visit (12 months); mean ± SD: 3.2 ± 0.5 and 11.8 ± 0.8 months, respectively. Samples were refrigerated during transport and stored at −80 °C until analysis. Whole genome DNA was extracted from 80 to 200 mg of stool using the QIAamp DNA Stool Mini Kit (Qiagen, Venlo, the Netherlands). The bacterial 16S

rRNA gene, hypervariable region V4, was amplified by PCR using universal bacterial primers: V4-515f: 5′- A ATGATACGGCGACCACCGAGATCTACAC TATGGTAATT GT GTGCCAGCMGCCGCGGTAA-3′, V4-806r:5′-CAAG CAGAAGACGGCATACGAGAT XXXXXXXXXXXX AGT CAGTCAG CC GGACTACHVGGGTWTCTAAT-3′.[35] The reverse primer was barcoded so that each sample could be uniquely identified post-sequencing (denoted in the primer sequence by Xs). Each PCR mixture (25 μL) contained 12.5 μL 2× Kapa2G Hotstart mix (Kapa Biosystems, Wilmington, MA), molecular biology reagent grade water (Sigma-Aldrich, St. Louis, MO, USA), 0.6 μM primer and 2 μL bacterial template DNA (5 ng/μL). PCR consisted of an initial DNA denaturation step (94 °C, 3 min), followed by 20 cycles of denaturation (94 °C, 30 s), annealing (50 °C, 30 s) and elongation (72 °C, 30 s), performed on a PTC-200 Thermal Cycler (MJ Research, St. Bruno, QC, Canada). Reactions were performed in triplicate and pooled with a negative control included in each run. 100 ng of product was condensed using an Amicon® Ultra-4 30K centrifugal filter (Millipore, Billerica, MA, USA), run through a 1.4% agarose gel, extracted and cleaned with the GENECLEAN® Turbo Kit (MP Biomedicals Inc, Solon, OH, USA).

16S rRNA sequencing and taxonomic classification

Pooled PCR amplicons were subjected to paired-end sequencing by Illumina MiSeq. Using a QIIME pipeline (v 1.6.0, qiime.org), forward and reverse reads were assembled using PandaSeq for a final length of 144 bp (unassemblable sequences discarded), demultiplexed and filtered against the GREENGENES reference database (v 12.10) to remove all sequences with < 60% similarity. Remaining sequences were clustered with Usearch61 at 97% sequence similarity against the GREENGENES database (closed-picking algorithm), and taxonomic assignment was achieved using the RDP classifier constrained by GREENGENES. Operational taxonomic units (OTUs) with overall relative abundance below 0.0001 were excluded from subsequent analyses. After cleaning and processing, a total of 110 million reads were retained (median 3.1×10^5 per sample, range 8.1×10^4–1.0×10^6), representing 1127 unique OTUs. For subsequent analyses, data were rarefied to 80 000 sequences per sample.

Determination of food sensitization

Allergy skin tests (epicutaneous) were performed using the Duotip-Test II (Lincoln Diagnostics Inc, Mississauga, ON, Canada) with the following food allergens (ALK-Abello, Mississauga, ON, Canada): cow's milk, egg white, soy and peanut. Histamine (1 mg/mL) was the positive control, and glycerine was the negative control. The largest wheal diameter and its orthogonal were measured 15 min after testing, and the wheal size was documented as the mean of these two measurements. A wheal size of 2 mm or greater than that elicited by the negative control was considered positive. Food sensitization was defined as a positive skin test response to one or more food allergen.

Statistical analysis

Distribution of potential confounders according to food sensitization status was assessed by Fisher's exact test. Using default settings in QIIME, OTU relative abundance was summarized at the phylum and family levels of taxonomy. Microbiota diversity within samples (alpha diversity at family level) was calculated using two standard metrics: the Chao1 estimator of OTU richness (which estimates the number of different OTUs present) and the Shannon diversity index (which evaluates both the number of OTUs and the evenness of their distribution). Microbiota community differences between samples (beta diversity) were tested by permutational multivariate analysis of variance (PERMANOVA) comparison of unweighted UNIFRAC [36] distance matrices, with 500 permutations. Median richness, diversity and relative abundance of dominant taxa were compared by non-parametric Kruskal–Wallis test and Spearman rank correlation. As others have done [25, 28, 29], we focused on dominant taxa to capture major trends and minimize multiple comparisons. As gut microbiota coexist in functional communities, ratios of specific taxa are commonly evaluated. We evaluated the ratio of Enterobacteriaceae to Bacteroidaceae (E/B ratio) as a measure of gut microbiota maturity as Proteobacteria (mainly Enterobacteriaceae) are prevalent in the early gut microbiota, while Bacteroidetes (mainly Bacteroidaceae) become dominant as the community matures towards an adult-like profile [37]. Associations with food sensitization were investigated by multiple logistic regression, with microbiota measures classified in quartiles and categorized as high (top quartile) or low (bottom quartile) to create dichotomous outcome variables (Table S2).

Sensitivity analyses were pursued to establish whether observed microbiota associations were upheld in two pre-defined subgroups. First, to ensure that microbiota differences preceded the onset of food sensitization, we excluded the two sensitized infants with pre-existing or unknown food allergy symptoms at the time of initial sampling at 3 months, leaving 10 sensitized infants (total $N = 164$; 154 controls vs. 10 sensitized infants) for analysis. Second, to address potential causes of microbiota differences, we excluded infants with major microbiota-disrupting exposures [24, 38] before initial sampling (i.e. caesarean delivery, antibi-

otic exposure or formula feeding), leaving $N = 38$ (34 controls vs. 4 sensitized infants).

Results

In this general population cohort of 166 infants, 12 (7.2%) were sensitized to one ($n = 9$) or more than one ($n = 3$) food allergen at 1 year of age, most commonly egg ($n = 9$) or peanut ($n = 4$) (Table 1). Of these 12 food-sensitized infants, 10 had no food allergy symptoms before the first faecal sample collection at 3 months of age, one had pre-existing symptoms (although sensitization was not confirmed by blood or skin testing), and symptoms at 3 months were unknown for one infant.

Infants with food sensitization at 1 year had significantly lower overall gut microbiota richness at 3 months (median Chao1 richness estimator: 25.0 vs. 28.0, $P = 0.02$) (Table 2). Gut microbiota diversity was also lower among sensitized infants at 3 months, but this difference was not statistically significant (median Shannon diversity index: 1.55 vs. 1.94, $P = 0.20$). At 1 year, richness and diversity did not differ between sensitized and non-sensitized infants ($P > 0.30$). Similar associations were found for incident sensitization and among a subgroup of 38 infants without major microbiota-disrupting exposures (i.e. those born vaginally, exclusively breastfed for at least 3 months and unexposed to antibiotics prior to sampling).

Gut microbiota composition at 3 months and 1 year differed by food sensitization status (Table 3, Fig. 1). Significant overall community differences at the OTU level of taxonomy were detected by PERMANOVA at 3 months (Pseudo$F = 1.52$, $P = 0.04$) and 1 year (Pseudo$F = 1.49$, $P = 0.03$). Among dominant microbial families, Enterobacteriaceae were substantially and significantly overrepresented among food-sensitized infants, at both 3 months (median relative abundance 46.4% vs. 17.3%, $P = 0.002$) and 1 year (6.4% vs. 1.0%, $P = 0.004$). Conversely, Bacteroidaceae were underrepresented (0.5% vs. 23.4%, $P = 0.09$ at 3 months; 19.1% vs. 45.6%, $P = 0.01$ at 1 year). Given these differences, we compared the ratio of Enterobacteriaceae to Bacteroidaceae (E/B ratio) between sensitized and non-sensitized infants, finding a significant difference at both sampling times (115.5 vs. 1.0, $P = 0.03$ at 3 months; 0.31 vs. 0.02, $P < 0.0001$ at 1 year). Similar associations were found for incident sensitization (Table S4), and in the subgroup of infants without major microbiota-disrupting exposures (Table S3). At 1 year, sensitized infants tended to have lower relative abundance of Ruminococcaceae (3.6% vs. 8.4%, $P = 0.10$); this difference reached statistical significance for incident sensitization ($P = 0.04$) and remained significant in the sub-

Table 1. Population characteristics and associations with food sensitization at 1 year

	Prevalence	Proportion with food sensitization* at 1 year	
	n (%)	*n* (%)	*P*
Sex			
Female	81 (48.8)	8 (9.9)	0.24
Male	85 (51.2)	4 (4.7)	
Birth mode			
Caesarean – elective	16 (9.6)	1 (6.3)	0.86
Caesarean – emergency	21 (12.7)	2 (9.5)	
Vaginal	129 (77.7)	9 (7.0)	
Self-reported maternal food allergy ($N = 164$)			
No	127 (77.4)	9 (7.1)	0.73
Yes	37 (22.6)	3 (8.1)	
Exclusive breastfeeding at 3 months ($N = 165$)			
No	82 (49.7)	3 (3.7)	0.13
Yes	83 (50.3)	9 (10.8)	
Solids introduced < 3 months ($N = 162$)			
No	153 (94.4)	11 (7.2)	1.00
Yes	9 (5.6)	0 (0.0)	
Antibiotic exposure by 3 months			
None	88 (53.0)	6 (6.8)	0.20
Indirect only (maternal intrapartum)	60 (36.1)	3 (5.0)	
Direct	18 (10.8)	3 (16.7)	
Antibiotic exposure by 1 year			
None	61 (36.7)	3 (4.9)	0.69
Indirect only (maternal intrapartum)	40 (24.1)	3 (7.5)	
Direct	65 (39.2)	6 (9.2)	
Older siblings ($N = 164$)			
No	79 (48.2)	7 (8.9)	0.56
Yes	85 (51.8)	5 (5.9)	
Furry Pets ($N = 154$)			
No	72 (46.8)	6 (8.3)	0.76
Yes	82 (53.2)	5 (6.1)	
Diagnosed food allergy before 3 months* ($N = 162$)			
No	156 (94.0)	10 (6.4)	0.35
Yes	6 (3.6)	1 (16.7)	
Unknown	4 (2.4)	1 (25.0)	
Rash before 3 months ($N = 162$)			
No	65 (40.1)	3 (4.6)	0.37
Yes	97 (59.9)	8 (8.2)	
Diagnosed atopic dermatitis at 1 year ($N = 165$)			
No	157 (95.2)	8 (5.1)	**< 0.001**
Yes	8 (4.8)	4 (50.0)	
Food sensitization at 1 year†			
No	154 (92.8)		
Yes	12 (7.2)		
Sensitization to:			
Peanut	4 (2.4)		
Milk	2 (1.2)		
Egg	9 (5.4)		
Soy	0 (0.0)		

*Parent reported.

†Food sensitization = positive skin prick test to one or more listed food allergen. See Methods for definitions of infant rash, maternal food allergy and maternal skin allergy. Comparisons by 2-sided Fisher exact test.

Table 2. Faecal microbiota richness and diversity at 3 months and 1 year of age, according to food sensitization at 1 year

	Microbiota at 3 months			Microbiota at 1 year		
Infants analysed Bodiversity metric	Non-sensitized Median (IQR)	Sensitized Median (IQR)	*P*	Non-sensitized Median (IQR)	Sensitized Median (IQR)	*P*
All infants	(*N* = 154)	(*N* = 12)		(*N* = 154)	(*N* = 12)	
Chao1 richness	28.0 (25.7–30.3)	25.0 (23.7–27.0)	**0.02**	34.9 (33.0–37.0)	36.2 (33.3–38.0)	0.30
Shannon diversity	1.94 (1.53–2.25)	1.55 (1.18–2.19)	0.20	2.24 (1.99–2.55)	2.29 (1.89–2.92)	0.63
Incident sensitization only*	(*N* = 154)	(*N* = 10)		(*N* = 154)	(*N* = 10)	
Chao1 richness	28.0 (25.7–30.3)	25.0 (23.9–26.0)	**0.03**	34.9 (33.0–37.0)	36.9 (33.3–38.2)	0.31
Shannon diversity	1.94 (1.53–2.25)	1.55 (1.14–2.48)	0.34	2.24 (1.99–2.55)	2.29 (1.93–2.87)	0.72
'Undisturbed' subgroup†	(*N* = 34)	(*N* = 4)		(*N* = 34)	(*N* = 4)	
Chao1 richness	28.2 (26.7–30.3)	24.8 (22.7–25.5)	**0.01**	34.8 (33.5–36.7)	34.7 (31.8–37.3)	0.57
Shannon diversity	1.82 (1.50–2.23)	1.22 (0.81–1.76)	0.09	2.26 (2.14–2.66)	2.47 (2.20–2.67)	0.63

Richness and diversity measures calculated at family level of taxonomy. Comparisons by nonparametric Kruskal–Wallis test.
IQR, interquartile range.
*Excludes sensitized infants with unknown or diagnosed food allergy before initial sampling at 3 months.
†Excludes children with major microbiota-disrupting exposures before initial sampling at 3 months (i.e. caesarean delivery, antibiotic exposure or complementary feeding).

Table 3. Relative abundance of dominant* phyla and families (italics) in faecal microbiota of infants at 3 months and 1 year of age, according to food sensitization at 1 year. (All infants; *N* = 166)

	Microbiota at 3 months				Microbiota at 1 year			
	Non-sensitized (*n* = 154)	Sensitized (*n* = 12)			Non-sensitized (*n* = 154)	Sensitized (*n* = 12)		
Dominant taxa*	Median (IQR)	Median (IQR)	*P*	FDRp	Median (IQR)	Median (IQR)	*P*	FDRp
Actinobacteria	4.6 (1.3–12.8)	7.0 (0.1–22.2)	0.71	0.94	1.3 (0.5–3.6)	3.3 (0.6–4.6)	0.42	0.56
Bifidobacteriaceae	4.5 (1.0–12.3)	7.0 (0.0–22.1)	0.87	0.95	1.3 (0.5–3.6)	3.2 (0.6–4.6)	0.50	0.56
Bacteroidetes	30.3 (0.2–62.3)	2.2 (0.2–20.8)	0.09	0.22	52.8 (43.4–64.7)	37.6 (13.4–53.9)	**0.02**	**0.05**
Bacteroidaceae	23.4 (0.1–54.8)	0.5 (0.1–7.7)	0.09	0.22	45.6 (30.5–56.9)	19.1 (0.2–42.0)	**0.01**	**0.04**
Firmicutes	24.0 (8.1–50.1)	21.5 (13.4–35.4)	0.66	0.94	34.2 (26.1–44.6)	33.6 (26.6–57.7)	0.63	0.63
Veillonellaceae	5.0 (0.8–17.6)	3.1 (0.5–13.9)	0.48	0.83	4.1 (1.4–10.2)	6.7 (0.7–15.2)	0.51	0.56
Lachnospiraceae	1.4 (0.1–7.4)	1.1 (0.0- 2.8)	0.34	0.68	13.3 (9.0–21.4)	18.2 (9.7–30.6)	0.37	0.56
Ruminococcaceae	0.1 (0.0–2.1)	0.1 (0.0–2.5)	0.95	0.95	8.4 (2.5–13.1)	3.6 (1.3–7.7)	0.10	0.24
Proteobacteria	18.0 (8.6–37.9)	46.4 (27.3–78.8)	**0.002**	**0.01**	4.5 (2.5–7.7)	8.3 (3.0–18.4)	0.21	0.38
Alcaligenaceae	0.0 (0.0–0.0)	0.0 (0.0–0.0)	0.86	0.95	1.6 (0.0–3.0)	0.1 (0.0–2.2)	0.22	0.38
Enterobacteriaceae	17.3 (7.7–36.7)	46.4 (27–78.2)	**0.002**	**0.01**	1.0 (0.3–3.2)	6.4 (2.1–7.7)	**0.004**	**0.02**
E/B Ratio	1.0 (0.2–170.5)	115.5 (7.6–318.0)	**0.03**	0.13	0.02 (0.01–0.09)	0.31 (0.11–17.27)	**0.0002**	**0.003**

IQR, interquartile range; E/B, Enterobacteriacea/Bacteroidaceae; FDR, false discovery rate.
*Dominant taxa have overall median relative abundance > 1% at 3 months and/or 1 year; phyla are in plain text and families are italicized. Comparisons by nonparametric Kruskal–Wallis test with FDR correction for multiple testing.

group of infants without major microbiota-disrupting exposures ($P = 0.02$). At both sampling times, relative abundance of class Clostridia was comparable among sensitized and non-sensitized infants ($P > 0.50$, data not shown). Correlations of individual taxon relative abundance with overall richness and diversity are shown in Table S5.

To further explore the association of food sensitization with the above measures of gut microbiota diversity and composition, we conducted multivariate logistic regression analyses (Table 4). At 3 months, each quartile increase in gut microbiota richness was associated with a 52% reduction in risk for food sensitization by 1 year (odds ratio (OR) 0.48, 95% confidence interval 0.25–0.90). Each quartile increase in E/B ratio was associated with a nearly twofold increase in risk for food sensitization (OR 1.89, 1.03–3.47). These associations were independent of each other (adjusted odds ratio (aOR) 0.45, 0.23–0.87 for richness; aOR 2.02, 1.07–3.80 for E/B ratio) (Fig. 2). At 1 year, gut microbiota richness was no longer associated with food sensitization (OR 1.24, 0.73–2.11); however, the strong association with gut microbiota composition remained (OR 4.43, 1.72–11.44 for each quartile increase in E/B

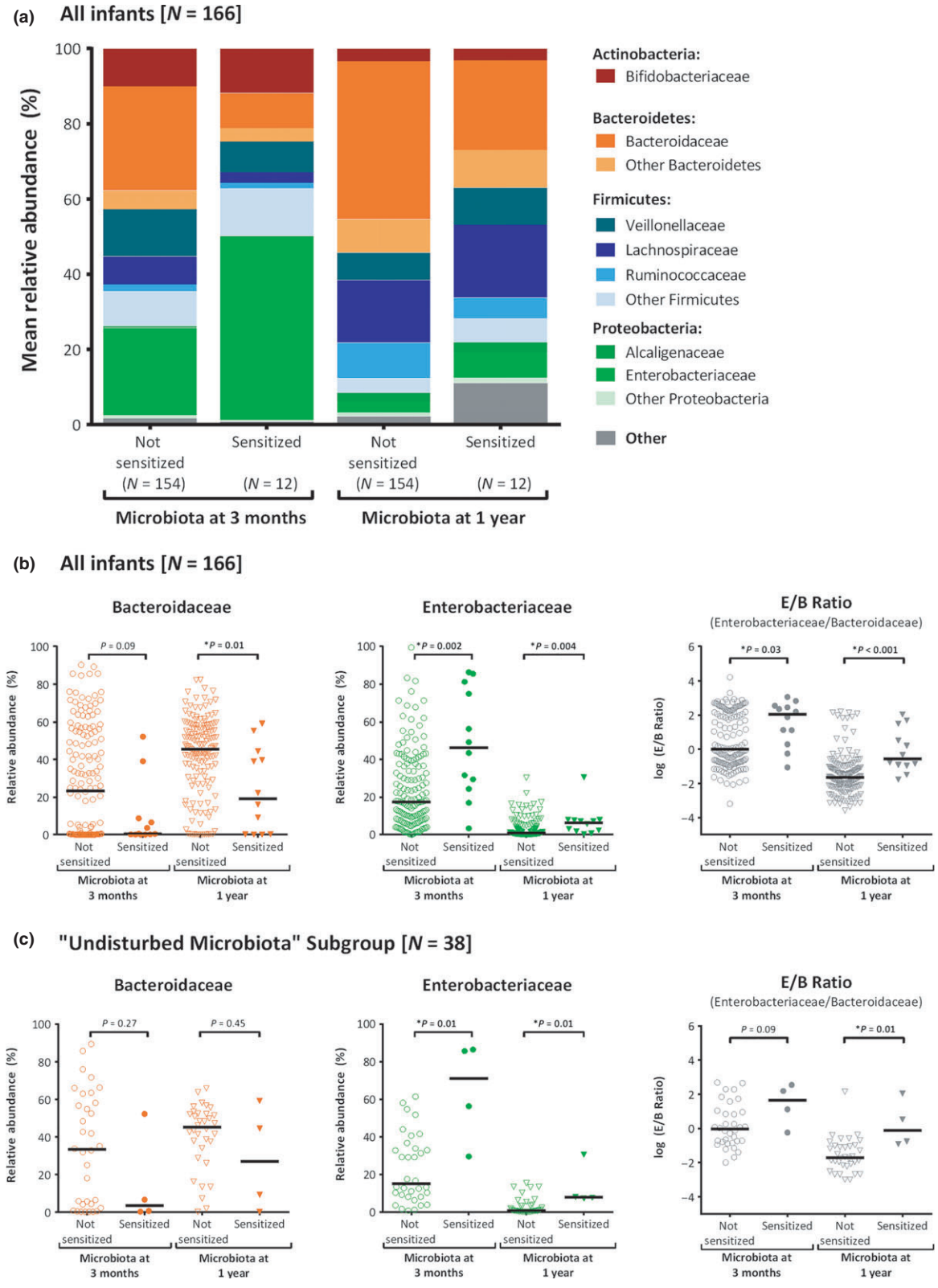

Fig. 1. Gut microbiota composition at 3 months and 1 year of age, according to food sensitization status at 1 year. (a) Mean relative abundance of dominant families (those with overall median relative abundance > 1% at either sampling time). (b, c) Relative abundance of Bacteroidaceae and Enterobacteriaceae, and log-transformed ratio of Enterobacteriaceae/Bacteroidaceae in all infants (b) or the 'undisturbed microbiota' subgroup (c: excludes children with major microbiota-disrupting exposures before initial sampling at 3 months; i.e. caesarean delivery, antibiotic exposure or complementary feeding). Bars indicate medians; comparisons by Kruskal–Wallis test.

Table 4. Crude and adjusted likelihood of food sensitization at 1 year according to key microbiota measures at 3 months and 1 year, with individual adjustments for major microbiota-disrupting exposures

Microbiota Measure Model Adjustments	Microbiota at 3 months OR (95% CI)	Microbiota at 1 year OR (95% CI)
E/B Ratio (per quartile increase)		
None: crude OR for food sensitization	1.89 (1.03–3.47)*	4.43 (1.72–11.44)*
Adjusted for antibiotic exposure†	2.00 (1.05–3.81)*	4.36 (1.69–11.26)*
Adjusted for caesarean delivery	1.98 (1.06–3.70)*	4.64 (1.75–12.33)*
Adjusted for exclusive breastfeeding at 3 months	1.73 (0.93–3.23)	4.36 (1.69–11.29)*
Low Ruminococcaceae (below vs. above median)		
None: crude OR for food sensitization	1.44 (0.44–4.72)	5.55 (1.18–26.16)*
Adjusted for antibiotic exposure†	1.44 (0.44–4.74)	5.82 (1.23–27.60)*
Adjusted for caesarean delivery	1.46 (0.44–4.80)	5.57 (1.18–26.25)*
Adjusted for exclusive breastfeeding at 3 months	1.05 (0.30–3.66)	5.45 (1.15–25.93)*
Chao1 Richness (per quartile increase)		
None: crude OR for food sensitization	0.48 (0.25–0.90)*	1.24 (0.73–2.11)
Adjusted for antibiotic exposure†	0.48 (0.25–0.90)*	1.22 (0.72–2.08)
Adjusted for caesarean delivery	0.48 (0.25–0.91)*	1.23 (0.72–2.11)
Adjusted for exclusive breastfeeding at 3 months	0.47 (0.25–0.89)*	1.16 (0.67–2.00)
Low Shannon Diversity (bottom quartile vs. others)		
None: crude OR for food sensitization	3.40 (1.03–11.21)*	1.58 (0.45–5.55)
Adjusted for antibiotic exposure†	3.42 (1.02–11.44)*	1.53 (0.43–5.40)
Adjusted for caesarean delivery	3.41 (1.02–11.39)*	1.59 (0.45–5.60)
Adjusted for exclusive breastfeeding at 3 months	2.78 (0.82–9.42)	1.99 (0.54–7.30)

OR, odds ratio; aOR, adjusted odds ratio; CI, confidence interval; E/B Ratio, ratio of Enterobacteriaceae/Bacteroidaceae relative abundance. Richness and diversity measured at family level.

*$P < 0.05$.

†Any antibiotic exposure before microbiota sampling (3 months or 1 year). All models are for $N = 166$, except those adjusted for breastfeeding, where $N = 165$ due to missing data for 1 non-sensitized infant.

ratio, OR 5.55, 1.18–26.16 for low Ruminococcaceae). Similar associations were found for incident sensitization (Table S6).

Sequential adjustment for major microbiota-disrupting exposures (antibiotic exposure, caesarean birth, exclusive breastfeeding at 3 months) revealed that associations of microbiota and food sensitization were generally independent of these early life events (Tables 4 and 5). Some associations were moderately attenuated with adjustment for breastfeeding. To minimize adjustments, and because we found no evidence of association with infant food sensitization (Table 1), regression models were not adjusted for maternal food allergy. Due to small group sizes, regression modelling was not feasible in the subgroup of infants without major microbiota-disrupting exposures; however, crude associations in this subgroup (Table 2 and Table S3) further demonstrated independence from birth mode, feeding and antibiotic exposure as the subgroup was homogeneous for these exposures.

Discussion

In a general population cohort of 166 Canadian infants, we found that food sensitization at 1 year was associated with several characteristics of the early gut microbiota. At 3 months, lower microbiota richness was associated with an increased likelihood of food sensitization by 1 year, and each quartile increase in E/B ratio doubled the risk of sensitization. By 12 months of age, microbiota richness was no longer associated with food sensitization, but sensitized infants were identified by a higher E/B ratio and low Ruminococcaceae abundance. These associations were upheld in a sensitivity analysis that excluded infants with a prior food allergy diagnosis, suggesting that 3-month microbiota differences preceded food sensitization. While several studies have identified infant gut microbiota changes in advance of atopic dermatitis or sensitization to any allergen [25, 26, 28, 29], and one new report has described altered microbiota composition concurrent with food allergy [32], we are the first to report temporal patterns of gut microbiota dysbiosis and food sensitization.

Our findings show that low gut microbiota richness at 3 months preceded food sensitization at 1 year, whereas concurrent richness at 1 year was unassociated with food sensitization. Notably, these associations were independent of breastfeeding, caesarean delivery and antibiotic use, which are all known to reduce gut microbiota richness and diversity [22, 39–41]. Our results are consistent with evidence from Wang et al. and

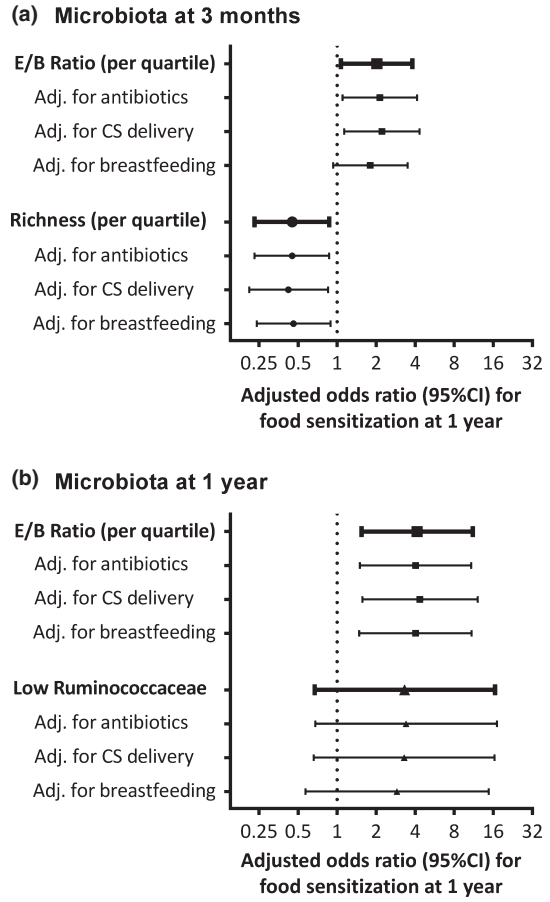

Fig. 2. Mutually adjusted† likelihood of food sensitization at 1 year according to key microbiota measures at 3 months and 1 year, with individual adjustments for major microbiota-disrupting exposures. Adj. = Adjusted; E/B Ratio, ratio of Enterobacteriaceae/Bacteroidaceae relative abundance; CS, caesarean section. †Mutually adjusted for two microbiota measures as shown: (a) E/B Ratio and Chao1 richness for microbiota at 3 months; (b) E/B Ratio and Low Ruminococcaceae (< median) for microbiota at 1 year. Antibiotics = any antibiotic exposure before microbiota sampling (3 months or 1 year). Breastfeeding = exclusive breastfeeding for at least 3 months. Chao1 richness measured at family level. All models are for $N = 166$, except those adjusted for breastfeeding, where $N = 165$ due to missing data for 1 non-sensitized infant.

Abrahamsson et al., where low microbiota diversity in early infancy (1 week and 1 month, respectively) was found to predict atopic dermatitis [25, 26]. Importantly, they also align with experimental evidence suggestive of causation, namely animal models showing altered gut immune responses when microbiota colonization is delayed [42], microbiota changes that precede gut inflammation [43], and enhanced food sensitization following microbiota disruption during the neonatal period [20]. Together with recent findings from Ling et al. and Nylund et al., where microbiota diversity later in infancy (5 and 6 months, respectively) was not associated with atopic disease [29, 32], our results suggest that *early* infancy is a critical period for microbiota development. These collective findings also illustrate the need for caution (and consideration of timing) when interpreting summary measures of richness and diversity for clinical applications.

We also report a novel association of prior and concurrent elevation in Enterobacteriaceae abundance among food-sensitized infants. Others have reported a similar association for atopic dermatitis (specific to caesarean-delivered infants) [44], and a cross-sectional study found increased Enterobacteriaceae in school children with various atopic conditions [45]. In contrast, Abrahamsson et al. and Ling et al. have reported lower abundance of Proteobacteria (the phylum containing Enterobacteriaceae) in atopic infants [25, 32]. This apparent contradiction may be a function of taxonomic resolution as Ling et al. [32] found that certain genera in this phylum were elevated (including the Enterobacteriaceae genus, *Escherichia/Shigella*). Similarly, Penders et al. found that *Escherichia coli* was detected more often in infants who subsequently developed atopic dermatitis (eczema) [28]. Infants with eczema are at higher risk for food sensitization [46]. While this may reflect genetic predisposition to atopic disease, it has also been proposed that eczema increases cutaneous absorption of food allergens through impaired skin barrier function [47]. Interestingly, the meconium (first stool) of infants born to mothers with eczema is enriched for Enterobacteriaceae [48], and there is evidence for maternal-infant transmission of gut microbiota including *E. coli* [49, 50]. Collectively, these findings suggest that elevated Enterobacteriaceae may be a marker for eczema, which could subsequently increase risk for food sensitization. We did not observe any distinct microbiota signatures among infants with atopic dermatitis in this cohort (not shown), but we intend to explore this hypothesis in a larger sample of infants following clinical assessment at 3 years of age.

Infants with food sensitization at age 1 also had a lower relative abundance of Bacteroidaceae and Ruminococcaceae. Members of these families stimulate the production and degradation of mucin, which is required to maintain an intact gut microbiota–mucin barrier [51]. Early deficiency of Bacteroidetes has been reported in infants with atopic dermatitis and food allergy [25, 29, 32] and is a predictable outcome of caesarean delivery [24, 39]. Breastfeeding promotes *Bacteroides* colonization as breast milk oligosaccharides are more efficiently metabolized by these species than other gut microbes [52]. As recently shown in a murine model of faecal transplantation, a gut microbiota abundant in *Bacteroides* contributes to prevention of milk allergy [53]. Little is known about Ruminococcaceae, aside from their ability to degrade fibre [54] and their greater presence in weaned or formula-fed infants [55, 56].

However, they are still detected in breastfed infants and interestingly, the extent of colonization varies according to the oligosaccharide content of breast milk [57]. Noteworthy in our study, food sensitization at age 1 was more likely in infants with low levels of Ruminococcaceae, an association that persisted following adjustment for birth method, breastfeeding and antibiotic exposure.

The association of food sensitization with Enterobacteriaceae and Bacteroidaceae was particularly evident when the ratio of these taxa was evaluated. The E/B ratio could be considered an indicator of gut microbiota maturity, as normal development involves early colonization by facultative anaerobes (predominantly Enterobacteriaceae), which deplete initial oxygen supplies to create a favourable environment for subsequent colonization by anerobes including Bacteroidaceae [37]. Thus, the E/B ratio is expected to decline with age as relative proportions of Enterobacteriaceae decline and Bacteroidaceae become more dominant, reflecting maturation toward an adult-like gut microbiota [37, 58, 59]. Our finding that the E/B ratio is elevated among food-sensitized infants suggest that delayed maturation of the gut microbiota may be a predictor of atopic disease.

Our study has several strengths, including the use of high-throughput sequencing to profile infant gut microbiota in a longitudinal, population-representative cohort. Observed changes from 3 to 12 months, such as decreasing abundance of Enterobacteriaceae and increasing predominance of Bacteroidaceae, are consistent with observations in other birth cohorts [39, 60]. With prospective collection of data and faecal samples, we identified specific microbiota changes that appear to precede food sensitization. Previous cross-sectional studies [32] could not exclude the possibility that microbiota dysbiosis occurred as a result of food allergy-related diet modification [30, 61]. Finally, we used statistical modelling and sensitivity analyses to explore whether observed associations of microbiota and sensitization were attributable to microbiota-disrupting exposures in early life.

The study also had limitations. Close to 90% of infants with a parent report of physician-diagnosed food allergy in the first year of life were skin-test negative, although the specific food may not have been tested in our standardized panel of milk, egg, soy and peanut. Due to the uncertainty of food allergy diagnosis at this age, we evaluated food sensitization (determined by skin prick testing), and not physician-diagnosed food allergy. There is evidence that food sensitivity at 1 year predicts future atopic disease [4–9], although the relevance to food allergy remains under investigation. As part of the ongoing CHILD study, subjects will be followed for the persistence of food sensitization and clinical food allergy diagnosis, as well as asthma onset. Second, while advantageous for addressing temporality and maximizing generalizability, our study design led to a relatively low (though population-representative) prevalence of food sensitization. While we were able to detect microbiota differences, the sample size was insufficient to allow simultaneous adjustment for multiple covariates, and larger studies are needed to confirm our findings. Third, while our results and careful sensitivity analyses suggest a causal association, we cannot exclude the possibility that microbiota disruption and food sensitization were separately caused by a common host or environmental factor. Earlier sampling of the faecal microbiota could provide additional insight into these mechanisms and associations. Finally, while high-throughput sequencing allows for complete and unbiased detection of gut microbiota, this technology lacks sensitivity for identifying differences among individual species [62]. As with all faecal microbiota studies, it is also necessary to consider that microbiota colonizing the gut mucosa may not be accurately reflected by the communities observed in stool, although Centanni et al. [63] have recently reported that (contrary to adults) the phylogenetic structures of faecal and enterocyte-associated microbiota are remarkably similar in infants.

In conclusion, we have shown that an elevated E/B ratio and low gut microbiota richness in early infancy are associated with subsequent food sensitization. At 1 year of age, high E/B ratio and low Ruminococcaceae abundance were strong markers for food sensitization. These findings suggest that gut colonization during infancy may influence the development of food allergy and atopic disease and could present novel targets for intervention. Further research, including planned follow up of the CHILD cohort, is required to fully characterize and establish the determinants of early gut microbiota dysbiosis and to confirm a causal association with atopic disease.

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Conflict of interests

The authors declare no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Population characteristics according to selection for the current analysis.

Table S2. Cutoffs for microbiota measure quartiles.

Table S3. Relative abundance of dominant phyla and families (italics) in fecal microbiota of infants at 3 months and 1 year of age, according to food sensitization at 1 year. ("Undisturbed gut microbiota" sub group‡; $N = 38$.).

Table S4. Relative abundance of dominant phyla, and families (italics) in fecal microbiota of infants at 3 months and 1 year of age, according to food sensitization at 1 year. (Incident food sensitization only; $N = 164$).

Table S5. Correlation of microbiota richness or diversity with relative abundance of dominant taxa, at 3 months and 1 year (all infants: $N = 166$).

Table S6. Crude and adjusted likelihood of food sensitization at 1 year according to key microbiota measures at 3 months and 1 year, with individual adjustments for major microbiota-disrupting exposures. (Incident food sensitization only; $N = 164$).