Establishment of the intestinal microbiota and its role for atopic dermatitis in early childhood

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Background: Perturbations in the intestinal microbiota may disrupt mechanisms involved in the development of immunologic tolerance. The present study aimed to examine the establishment of the infant microbiota and its association to the development of atopic dermatitis (AD).

Methods: Within a randomized, placebo-controlled trial on the prevention of AD by oral supplementation of a bacterial lysate between week 5 and the end of month 7, feces was collected at the ages of 5 weeks (n = 571), 13 weeks (n = 332), and 31 weeks (n = 499) and subjected to quantitative PCRs to detect bifidobacteria, bacteroides, lactobacilli, *Escherichia coli*, *Clostridium difficile*, and *Clostridium* cluster I.

Results: Birth mode, breast-feeding but also birth order had a strong effect on the microbiota composition. With increasing number of older siblings the colonization rates at age 5 weeks of lactobacilli ($P < .001$) and bacteroides ($P = .02$) increased, whereas rates of clostridia decreased ($P < .001$). Colonization with clostridia, at the age of 5 and 13 weeks was also associated with an increased risk of developing AD in the subsequent 6 months of life (odds ratio$_{adjusted}$ = 2.35; 95% CI, 1.36-3.94 and 2.51; 1.30-4.86, respectively). Mediation analyses demonstrated that there was a statistically significant indirect effect via *Clostridium* cluster I colonization for both birth mode and birth order in association to AD.

Conclusion: The results of this study are supportive for a role of the microbiota in the development of AD. Moreover, the "beneficial" influence of older siblings on the microbiota composition suggests that this microbiota may be one of the biological mechanisms underlying the sibling effect. (J Allergy Clin Immunol 2013;■■■:■■■-■■■.)

Key words: Microbiota, atopic dermatitis, birth mode, siblings, mediation analysis

The intestinal microbiota is a key source of immune development and regulation early in life. Deprivation of microbial exposure is thought to predispose to immune dysregulation and the development of atopic diseases.[1] Animal studies have found that oral tolerance is difficult to achieve in germ-free animals[2] and that administration of lipopolysaccharides (constituents of the outer membrane of gram-negative bacteria) together with food antigens increases the tolerizing effect of feeding.[3] In addition, a complex intestinal microbiota, rather than colonization with a single microorganism, seems to be required to support oral tolerance development.[4]

Numerous epidemiologic studies showed indeed that the microbiota of infants with allergies differs from the microbiota of infants without allergies.[5] Although most of these studies were case–control studies, some, but not all, of the longitudinal studies found that these differences in the composition and diversity of the microbiota actually preceded the development of allergic manifestations.[5-7] Thus, the immune modulation by gastrointestinal (GI) microbiota is still one of the key candidates that may explain the increase of allergies (and other immune disorders) in terms of the hygiene hypothesis.

The fetal intestine is sterile and bathed in swallowed amniotic fluid. After delivery, the colonization of the intestines by a variety of microorganisms begins.[8] Intestinal colonization involves a succession of bacterial populations waxing and waning as the diet changes and the host develops.[9]

Factors that influence the intestinal microbiota composition can be divided into host factors (such as pH, bile acids, pancreatic enzymes, mucus composition, and transit time), nonhost factors (such as diet, medication, and environmental factors), and bacterial factors (such as adhesion capacity, enzymes, and metabolic capacities).[10] Especially changes in nonhost factors due to Western lifestyle (antibiotic use, diet, smaller family sizes, increased hygiene) may result in perturbations in the GI microbiota composition and thus may interfere with the mechanisms involved in the development of immunologic tolerance.[11]

In the present study, we investigated the influence of nonhost factors on the establishment of the intestinal microbiota in infancy, within a randomized, placebo-controlled trial of primary prevention of atopic dermatitis (AD) by oral supplementation of a bacterial lysate in very early infancy. Furthermore, we prospectively examined the composition of the infant intestinal

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Abbreviations used
AD: Atopic dermatitis
C-section: Cesarean section
GI: Gastrointestinal
OR: Odds ratio
CFU: Colony-forming units

microbiota in association to the subsequent development of AD and sensitization to common food allergens.

METHODS

Study population

The present study was conducted within the context of a randomized, placebo-controlled trial (registration no. ISRCTN60475069) on the primary prevention of AD by an orally applied bacterial lysate that contained heat-killed *Escherichia coli* Symbio DSM 17252 and *Enterococcus faecalis* Symbio DSM 16440 (Pro-Symbioflor). The study was approved by the Charité Ethics Committee in 2002, and all parents gave informed consent. The design of this trial has been described in detail elsewhere.[12]

Briefly, 606 healthy newborns (at term and birth weight ≥ 2500 g) with a single or double heredity for atopy (AD, allergic rhinitis, and/or asthma) were included in the study. Exclusion criteria were antibiotic treatment or other medication directly after birth, lymphocytopenia or thrombocytopenia, intensive care after birth, or parents lacking knowledge of the German language.

After an initial screening phase (age birth to 4 weeks), enrolled infants were randomly assigned at 4 to 5 weeks of age. From week 5 until the end of week 31 postpartum, infants were orally supplemented with the bacterial lysate or placebo daily.

Parents were asked to sample the infant's feces at the age of 5 weeks (start of intervention; n = 571), at 13 weeks (in a random subgroup only; n = 332), and at 31 weeks (end of intervention period; n = 499). Participants were provided with standard stool tubes with spoons attached to the lid (Sarstedt, Hilden, Germany) and were instructed to collect the fecal sample before the next visit during which times samples were handed to the researchers.

During the intervention period and thereafter until the age of 3 years, children were clinically examined at a regular basis by a pediatrician for signs of AD.

DNA purification from feces

At the laboratory 1 spatula of feces (approximately 200 mg) was diluted in 2 mL of Crowser-Medium (5 g of Lab Lemco [meat extract 3.0 g/L and Pepton 5 g/L] + 50 mL of Gycerol and 450 H_2O; ~pH 7.3) and stored at −80°C until further analysis.

For DNA isolation, 0.2 mL of the diluted feces was added to a 2-mL vial that contained approximately 300 mg of glass beads (diameter, 0.1 mm) and 1.4 mL of ASL buffer from the QIAamp DNA stool minikit (Qiagen, Hilden, Germany), and the samples were disrupted in a mechanical bead beater at 5000 rpm for 3 minutes. Subsequently, the bacterial DNA was isolated from the samples with the QIAamp DNA stool mini kit, according to the instructions provided by the manufacturer. The DNA was eluted in a final volume of 200 μL. DNA yields (ng/μL) were measured with an Eppendorf Photometer.

Microbial analysis of fecal samples

DNA from the fecal samples was subjected to quantitative real-time PCR assays for the quantification of bifidobacteria, *E coli*, *Clostridium difficile*, *Clostridium* cluster I (*Clostridium sensu stricto*), *Bacteroides fragilis* group, and lactobacilli targeting 16S rDNA gene sequences (see Table E1 for primer and probe sequences in this article's Online Repository at www.jacionline.org) as described previously.[13]

Counts of the bacterial groups and species were calculated for each stool sample from the threshold cycle values by using constructed standard curves and were expressed as the $\log_{10}$ colony-forming units (CFU) per milliliter of diluted feces. The prevalence of colonization was expressed as the percentage of infants colonized with a specific bacterial group or species.

Diagnosis of AD

Infants were clinically examined by a pediatrician during the intervention phase at the ages of 13, 21, and 31 weeks (end of the intervention phase). In the follow-up phase, participants were seen for additional visits at 1, 2, and 3 years of age. AD was clinically assessed.

Sensitization to food allergens

Sensitization to common food allergens (soy, peanut, cow's milk, hen's egg, wheat, and cod fish) was tested by panel ImmunoCAP fx5 on blood samples taken at 31 weeks, 1 year, and 2 years of age. Children who tested positive to any of the food allergens (>0.35 IU/mL) at any time point were labeled sensitized. Children were regarded nonsensitized when they were tested at least at age 2 years and were found negative at this time point and were negative at the other time points at which they were tested (31 weeks and/or 1 year).

Statistical analysis

Effects of birth characteristics, environmental factors, and intervention on gut microbiota. The following potential determinants were examined in association to the GI microbiota at the age of 5 and 13 weeks: sex (male/female), birth mode (spontaneous vaginal, assisted vaginal [forceps/vacuum extraction], cesarean section [C-section]), number of siblings (0, 1, 2 or more), atopy mother, or atopy father. For GI microbiota at the age of 31 weeks, this list was complemented with duration of breast-feeding (0-3 months, 3-6 months, or >6 months) and day care attendance (group size ≥3 children) during the first 6 months of life.

The Mann–Whitney rank sum test was used for the associations between these determinants and the counts of the bacteria under study (including the noncolonized infants with counts defined as zeros). The same method was used to examine the influence of the intervention on the bacterial counts in those infants who completed follow-up until the end of the treatment (age 31 weeks).

GI microbiota composition in association with AD and sensitization. Logistic regression analyses were used to test for associations between colonization with gut bacteria (colonized or noncolonized) under study and the development of AD or sensitization to food allergens respectively.

The following covariates were taken into account in the logistic regression models: sex, birth weight, maternal and paternal atopy, (duration of) breast-feeding, number of siblings, mode of delivery, and treatment group (placebo vs active group).

Logistic regression analyses were also used for associations between the concentrations (counts) of the gut bacteria and AD. Here, we additionally adjusted for the DNA concentrations of the samples to normalize the data. To test for trend bacterial counts were categorized (noncolonized infants were used as a reference category, and the remaining colonized infants were accommodated in 3 equal groups). These analyses were all limited to the completers group for the specific end points.

Survival analysis by Cox regression was used to examine the effects of the GI microbial composition on AD-free survival time.

Follow-up time for subjects who developed AD was calculated as the number of days between birth and the date of the visit at which AD was first diagnosed. The follow-up time of children who had not developed AD (yet) until they were lost to follow-up during the follow-up was the age in days at the moment of the last performed study visits.

To check the potential effect-modifying role of the treatment groups (placebo vs active group), we initially incorporated interaction terms between the variable "treatment group" and the variables for the different bacteria under study in all statistical models. Because none of these interaction terms appeared statistically significant, they were removed from the models, and

TABLE I. Baseline characteristics of both treatment groups in the entire study population and the present study population

Baseline characteristics	Total study population		Present study population*	
	Active (n = 303)	Placebo (n = 303)	Active (n = 285)	Placebo (n = 286)
Age of newborns (wk), median (25%-75% quartile)	5.1 (4.6-5.7)	5.1 (4.6-5.7)	5.1 (4.6-5.7)	5.1 (4.6-5.7)
Proportion males, no. (%)	161 (53.1)	152 (50.2)	155 (54.4)	140 (49.0)
Weight at birth (g), median (25%-75% quartile)	3480 (3140-3780)	3480 (3230-3800)	3480 (3140-3790)	3495 (3230-3800)
Gestational age (wk), median (25%-75% quartile)	40 (39-40)	40 (38-40)	40 (39-40)	40 (38-40)
Cesarean section, no. (%)	75 (24.8)	76 (25.1)	70 (24.6)	74 (26.0)
Breast-fed				
Never, no. (%)	9 (3.0)	6 (2.0)	7 (2.5)	5 (1.7)
<3 mo, no. (%)	59 (19.5)	41 (13.5)	57 (20.0)	36 (12.6)
3-6 mo, no. (%)	38 (12.5)	38 (12.5)	34 (11.9)	36 (12.6)
6-9 mo, no. (%)	71 (23.4)	81 (26.7)	67 (23.5)	78 (27.3)
9-12 mo, no. (%)	63 (20.8)	60 (19.8)	60 (21.1)	58 (20.3)
>12 mo, no. (%)	63 (20.8)	77 (25.4)	60 (21.1)	73 (25.5)
No. of siblings, median (25%-75% quartile)	1 (1-1)	1 (1-1)	1 (1-1)	1 (1-1)
Frequency of siblings				
1 sibling, no.	122	110	110	99
2 siblings, no.	22	28	21	22
3 siblings, no.	5	3	5	3
4 siblings, no.	—	—	—	—
5 siblings, no.	—	1	—	1
Smoking mother				
Before pregnancy, no. (%)	81 (26.7)	73 (24.1)	77 (27.0)	67 (23.4)
During pregnancy, no (%)	75 (24.8)	66 (21.8)	71 (24.9)	62 (21.7)
After pregnancy, no. (%)	71 (23.4)	74 (24.4)	69 (24.2)	70 (24.5)
Family history of atopy				
Both parents, no. (%)	148 (49.2)	157 (52.5)	137 (48.1)	148 (51.7)
One of both parents, no. (%)	154 (50.5)	145 (47.2)	147 (51.6)	137 (47.9)
Mother, no. (%)	79 (25.7)	85 (27.4)	76 (26.7)	79 (27.6)
Father, no. (%)	75 (24.8)	60 (19.8)	71 (24.9)	58 (20.3)
Single mother	2 (0.6)†	1 (0.3)‡	1 (0.4)‡	1 (0.3)‡
Underlying parental disease§				
Mother				
Atopic eczema	117 (38.7)	109 (36.1)	110 (38.6)	105 (36.7)
Allergic rhinitis	171 (56.6)	194 (64.2)	163 (57.2)	181 (63.3)
Allergic asthma	89 (29.5)	99 (32.8)	83 (29.1)	92 (32.2)
Father				
Atopic eczema	55 (18.2)	65 (21.5)	52 (18.4)	64 (22.5)
Allergic rhinitis	204 (67.5)	189 (62.6)	189 (66.5)	179 (62.8)
Allergic asthma	70 (23.2)	67 (22.2)	63 (22.1)	64 (22.5)

*All children with fecal samples collected at age 5 weeks.
†Family history of atopy was unknown for 1 father but known for the second father.
‡Family history of atopy was unknown for the father.
§Underlying diseases in parents ranged between 1 and 3.

associations are reported for the entire study population without stratification for treatment group.

Mediation analyses. To investigate whether *Clostridium* cluster I mediated the associations between birth mode, respectively, birth order and AD, we used the *ab* product-coefficient method.[14] This entails estimating the product of 2 coefficients: that of the association between birth mode/siblings and *Clostridium* cluster I (the *a* path) and that of the association between *Clostridium* cluster I and AD (the *b* path). Standardized coefficients and standard errors were obtained from these analyses. To test for statistical significance of the *ab*-product coefficient, the Sobel test was used.

RESULTS

At the start of the intervention there were neither differences between the intervention groups for baseline characteristics nor differences between baseline characteristics of the entire study population (n = 303 in both the active and placebo groups) and the study population that was included for the present study (those children of whom fecal samples were collected at baseline; n = 285 and n = 286 in the active and placebo group, respectively; Table I).

Effects of birth characteristics, environmental factors, and intervention on gut microbiota

A strong association between birth by C-section and the GI microbiota composition was found: infants delivered by C-section were less often colonized by bifidobacteria, bacteroides, and *E coli*, but more frequently colonized by both *Clostridium* cluster I and *C difficile*. If colonized, infants delivered by C-section had also lower counts (CFU/mL diluted feces) of bifidobacteria and bacteroides and a higher count of clostridia than infants delivered spontaneously (Table II). At the age of 13 weeks and even at the age of 31 weeks the effects of C-section were still prominent, with a reduced prevalence of colonization by bacteroides and a slightly higher prevalence of colonization by *Clostridium* cluster I. Furthermore, at 31 weeks

TABLE II. Median counts and prevalence of colonization with selected gut bacteria in feces of infants at age 5 (n = 571), 13, and 31 weeks (n = 499)

	No.	Bifidobacteria, counts* (%)	*Clostridium* cluster I, counts* (%)	*C difficile*, counts* (%)	Lactobacilli, counts* (%)	*B fragilis* group, counts* (%)	*E coli*, counts* (%)
Age 5 wk							
Birth weight							
<3000 g	78	8.46 (80.8)†	5.58 (50.0)	7.47 (25.6)	6.15 (19.2)	9.66 (41.0)†	8.72 (55.1)
3000-4000 g‡	421	8.68 (90.7)	5.65 (42.5)	6.98 (24.0)	6.07 (19.7)	9.40 (57.0)	8.46 (61.5)
≥4000 g	72	8.82 (90.3)	5.44 (49.3)	5.06 (19.4)	6.28 (26.4)	9.44 (70.8)	8.50 (75.0)
Delivery							
Spontaneous‡	391	8.79 (90.5)	5.54 (37.2)	6.55 (19.4)	6.03 (22.0)	9.48 (65.0)	8.45 (67.3)
Assisted vaginal	34	8.38 (97.1)	6.28 (38.2)	6.88 (41.2)§	6.89 (8.8)	9.80 (79.4)	8.69 (64.7)
C-section	144	8.33 (84.0)§	5.62 (65.3)§	7.48 (31.3)§	6.28 (19.4)	7.01 (29.2)§	8.63 (48.6)§
No. of siblings							
0‡	310	8.57 (85.8)	5.72 (52.3)	6.84 (26.1)	6.15 (15.8)	9.42 (53.9)	8.60 (59.0)
1	209	8.76 (95.2)§	5.44 (37.0)§	7.01 (22.0)	6.03 (24.9)†	9.44 (56.0)	8.17 (65.6)
≥2	52	8.80 (86.5)	5.41 (26.9)§	5.18 (15.4)	7.11 (30.8)§	9.34 (75.0)†	8.48 (69.2)
$P_{for\ trend}$			.001	.046	.001	.02	
Age 13 wk							
Birth weight							
<3000 g	44	8.91 (86.4)	6.19 (65.9)	7.18 (36.4)†	7.11 (25.0)	10.21 (50.0)	9.00 (68.2)
3000-4000 g‡	252	9.03 (89.7)	5.92 (51.0)	6.93 (20.9)	6.93 (26.5)	9.85 (54.5)	8.99 (76.6)
≥4000 g	35	9.20 (94.3)	5.52 (34.3)†	6.64 (20.0)	7.42 (37.1)	9.80 (62.9)	9.05 (82.9)
$P_{for\ trend}$			.001				
Delivery							
Spontaneous‡	232	9.02 (90.1)	5.73 (46.8)	6.88 (19.3)	7.00 (26.2)	9.85 (60.1)	9.00 (80. 2)
Assisted vaginal	17	9.42 (88.2)	6.11 (52.9)	7.04 (29.4)	7.06 (35.3)	10.39 (64.7)	9.04 (64.7)
C-section	83	8.91 (89.2)	6.19 (63.9)§	7.17 (32.5)†	6.88 (28.9)	10.05 (37.3)§	8.98 (67.5)
No. of siblings							
0‡	198	8.99 (88.4)	6.03 (58.8)	7.04 (23.6)	7.04 (21.6)	9.87 (52.3)	9.03 (73.7)
1	102	9.03 (92.2)	5.75 (44.1)§	6.89 (26.5)	6.91 (36.3)†	9.89 (54.9)	8.97 (81.4)
≥2	32	9.32 (90.6)	5.93 (28.1)§	7.99 (9.4)	7.00 (34.4)	9.75 (68.8)	9.02 (75.0)
$P_{for\ trend}$			.001				
Age 31 wk							
Birth weight							
<3000 g	66	8.91 (90.9)	5.33 (78.8)	7.31 (53.0)†	6.31 (43.9)	10.38 (56.1)	8.84 (90.9)
3000-4000 g	371	9.04 (94.6)	5.51 (74.1)	6.99 (39.1)	6.31 (46.1)	10.07 (68.7)	8.74 (88.9)
≥4000 g	62	9.22 (92.1)	5.36 (65.1)	6.03 (39.7)	6.35 (52.4)	10.04 (77.8)	8.77 (92.1)
Delivery							
Spontaneous‡	346	9.11 (93.6)	5.41 (72.5)	6.83 (39.9)	6.32 (49.1)	10.18 (74.0)	8.82 (91.0)
Assisted vaginal	32	9.11 (96.9)	5.16 (62.5)	7.32 (34.4)	6.27 (56.2)	10.29 (62.5)	9.09 (78.1)
C-section	119	8.87 (94.2)	5.60 (80.0)†	7.23 (45.8)	6.22 (37.5)†	9.80 (54.2)§	8.71 (88.3)
Breast-feeding							
0-3 mo‡	55	8.75 (92.7)	5.42 (83.6)	7.03 (78.2)	6.00 (40.0)	10.31 (83.6)	8.59 (100.0)
3-6 mo	39	8.91 (92.3)	5.60 (82.1)	6.97 (64.1)	6.09 (35.9)	10.22 (84.6)	8.53 (97.4)
≥6 mo	405	9.11 (94.1)§	5.42 (71.6)	6.75 (33.6)§	6.38 (48.6)†	10.04 (64.4)§	8.87 (87.4)
No. of siblings							
0‡	276	8.99 (94.9)	5.46 (74.4)	6.98 (40.4)	6.24 (40.4)	10.01 (61.7)	8.71 (87.7)
1	178	9.05 (93.3)	5.60 (72.5)	6.96 (43.3)	6.44 (51.7)†	10.19 (73.0)§	8.90 (91.6)†
≥2	45	9.42 (88.9)	5.07 (73.3)	7.33 (35.6)	6.30 (64.4)§	10.18 (88.9)§	8.92 (93.3)
$P_{for\ trend}$					.001	.001	

*Counts expressed as median (log_{10} CFU/mL feces). Counts were calculated from positive samples only.
†$P < .05$, as determined with the Mann–Whitney rank-sum test, calculated from all samples (the statistical significance refers to an overall difference incorporating counts, including noncolonized infants with counts being zero).
‡Reference category.
§$P < .01$, as determined with the Mann–Whitney rank-sum test, calculated from all samples (the statistical significance refers to an overall difference incorporating counts, including noncolonized infants with counts being zero).

of age approximately one-half of the children delivered spontaneously were colonized by lactobacilli, whereas this number was significantly lower (37.5%) in children delivered by C-section.

Next to the mode of delivery, the number of older siblings showed a strong association with the establishment of the GI microbiota. With increasing number of siblings the colonization rate of clostridia decreased ($P_{for\ trend} < .001$) and lactobacilli ($P_{for\ trend} < .001$) and bacteroides ($P_{for\ trend} = .02$) at the age of 5 weeks increased. The influence of older siblings on the microbiota composition persisted until the age of 31 weeks,

when increased prevalence of lactobacilli and bacteroides were found in children with 1 or ≥2 siblings compared with single children (Table II).

Multivariable analyses found that the influence of older siblings persisted after adjusting for mode of delivery and vice versa (see Table E2 in this article's Online Repository at www.jacionline.org), clearly showing that birth mode and sibling status both act independently on microbiota composition (also visually depicted in Fig E1, *A-F*, in this article's Online Repository at www.jacionline.org).

Infants with relatively low birth weight (2500-3000 g) were found to be less frequently colonized by bifidobacteria and bacteroides at 5 weeks of age, and they more frequently harbored *C difficile* at the ages of 13 and 31 weeks.

Although the high number of children who were initially breast-fed (>97%) did not allow studying the effect of breast-feeding on early infant microbiota, we were able to examine the effect of duration of breast-feeding on the GI microbiota at the age of 31 weeks. A longer duration of breast-feeding was associated with a lower prevalence of *C difficile* and bacteroides, whereas the prevalence of colonization by lactobacilli and (to a lesser extent) bifidobacteria were increased when breast-feeding was continued for 6 months or longer. The microbiota composition was not associated with sex, day care attendance, and maternal and paternal atopy at any of the studied ages. The influence of all these factors on the GI microbiota at all ages is summarized in Table E3 (in the Online Repository at www.jacionline.org).

A total of 526 infants completed the intervention to 31 weeks of age as per protocol. The intervention had no influence on the microbiota composition because neither the prevalence nor the abundance of the studied bacteria differed between the study groups at the end of the intervention phase (see Table E4 in this article's Online Repository at www.jacionline.org).

GI microbiota composition in association to AD and sensitization to food allergens

Colonization with *Bifidobacterium* spp, *E coli, C difficile, B fragilis* group, and *Lactobacillus* spp at the ages of 5, 13, and 31 weeks was not associated with the development of AD. However, colonization with members of *Clostridium* cluster I at age 5 weeks was associated with an increased risk of developing AD before the age of 31 weeks (Table III). This association showed a dose-response relationship ($P_{for\ trend}$ = .002). Moreover, this association persisted for AD development up to the age of 2 years (odds ratio $[OR]_{adjusted}$, 1.67; 95% CI, 1.05-2.64; P = .03) and, although no longer statistically significant, the age of 3 years (OR, 1.52; 95% CI, 0.95-2.41; P = .08).

When we stratified our analyses on the initial intervention arms (data not shown), similar results were found in the placebo group and the active group of the original intervention study.

In accordance, survival analysis also showed an increased risk of *Clostridium* cluster I colonization on AD development (adjusted hazard ratio = 1.49; 95% CI 1.05-2.11; see Fig E2 in this article's Online Repository at www.jacionline.org).

Colonization with *Clostridium* cluster I at the age of 13 weeks was still associated with an increased risk of developing AD ($OR_{adjusted}$, 2.51; 95% CI, 1.30-4.86; see Table E5 in this article's Online Repository at www.jacionline.org), whereas the microbiota at the age of 31 weeks was no longer associated with AD development.

TABLE III. Associations (adjusted) between colonization with the gut bacteria at age 5 weeks and the development of AD between age 5 and 31 weeks (n = 497)

	AD		
	Prevalence, % (n/N)	OR (95% CI)	$OR_{adjusted}$ (95%CI)*
Bifidobacterium spp			
No†	13.5 (7/52)	1.0	1.0
Yes	18.7 (83/445)	1.44 (0.63-3.31)	1.40 (0.56-3.47)
E coli			
No†	16.2 (31/191)	1.0	1.0
Yes	19.3 (59/306)	1.24 (0.77-2.00)	1.12 (0.66-1.90)
C difficile			
No†	17.2 (66/383)	1.0	1.0
Yes	21.1 (24/114)	1.28 (0.76-2.15)	1.24 (0.70-2.19)
B fragilis group			
No†	18.4 (40/217)	1.0	1.0
Yes	17.9 (50/280)	0.97 (0.61-1.53)	0.99 (0.57-1.71)
Lactobacillus spp			
No†	17.4 (68/390)	1.0	1.0
Yes	20.6 (22/107)	1.22 (0.71-2.09)	1.25 (0.69-2.25)
Clostridium cluster I			
No†	14.5 (40/276)	1.0	1.0
Yes	22.7 (50/220)	1.74 (1.10-2.75)‡	2.32 (1.36-3.94)‡

OR, Odds ratio.
*Analyses were adjusted for sex, birth weight, maternal and paternal atopy, (duration of) breast-feeding, number of siblings, mode of delivery, and treatment group.
†Reference category.
‡P < .05.

Sensitivity analyses, excluding infants who were treated with antibiotics (n = 15) or who had experienced gastroenteritis (n = 13, of whom 2 were also treated with antibiotics) within 1 month before fecal sampling at age 31 weeks did not change these results.

Sensitization to food allergens was not associated with any of the bacteria under study at ages 5, 13, or 31 weeks (data not shown).

Mediation analyses

Because both birth mode and birth order strongly influenced *Clostridium* cluster I colonization, and this bacterial group was also associated with the development of AD, colonization by *Clostridium* cluster I might act as an intermediate factor in the association between birth mode, respectively, and birth order and AD. Although the direct effect of both birth mode and birth order on AD appeared not to be statistically significant, mediation analyses (Table IV) demonstrated that there was a significant indirect effect via *Clostridium* cluster I colonization for both birth mode and birth order as indicated by the statistically significant *ab* cross-products.

DISCUSSION

Within the present study, we examined the influence of environmental determinants on the establishment of the infant microbiota composition and subsequently investigated the role of this microbiota composition in association with the development of AD.

This study was conducted within the context of a randomized, placebo-controlled trial on the primary prevention of AD in

TABLE IV. Mediation by *Clostridium* cluster I colonization at age 5 weeks in the association between birth mode or older siblings and AD

		a Path*		*b* Path†		Mediated effect‡	
Dependent	Independent	Coefficient	SE	Coefficient	SE	*ab*	*P* value
AD	C-section§	0.3167	0.052	0.1655	0.068	0.0165	.02
AD	Older siblings‖	−0.185	0.050	0.1634	0.065	−0.0092	.04

*Standardized coefficient of the association between mode of delivery/older siblings and Clostridium Cluster I.
†Standardized coefficient of the association between Clostridium Cluster I and AD (note that the *b* path is also adjusted for mode of delivery/older siblings).
‡Product of the standardized coefficients of the *a* and *b* paths (mediated effect) and *P* value derived from the Sobel test.
§Reference category: vaginal delivery.
‖Reference category: no older siblings.

high-risk newborns by oral supplementation of a bacterial lysate of *E coli* and *E faecalis*.[12] The allocated intervention did not influence the microbiota composition, which was as expected because the intervention did not contain living bacteria.

Despite that almost all children in our study were breast-fed, we found profound differences in the microbiome composition, at the age of 31 weeks, of infants who were breast-fed for >6 months compared with infants who were breast-fed for a shorter period. In the past decades formulas have become supplemented with compounds such as long-chain polyunsaturated fatty acids, oligosaccharides, nucleotides, and lactoferrin to mimic human milk as much as possible. Yet, numerous bioactive compounds in breast milk, including immunoglobulins, cytokines, hormones, enzymes, and microbes, are not present in infant formulas that might contribute to the beneficial effect of breast-feeding on microbiota development.

Cesarean delivery had a strong effect on the infant microbiota, especially the decreased colonization rate of bacteroides and the increased prevalence of clostridia, including *C difficile*, that persisted over time. The findings are in agreement with several previous studies on this subject.[7,13,15] A recent study that used next-generation sequencing to characterize the maternal and infant microbiota indeed confirmed that vaginal-delivered infants acquired bacterial communities resembling their own mothers' vaginal microbiota. However, infants delivered by C-section harbored bacterial communities that were most similar to those found on the skin surface.[16]

A striking resemblance was observed between the colonization pattern of firstborns and infants born by C-section with a higher colonization rate of *C difficile* and other clostridia and lower rates of lactobacilli, bifidobacteria, bacteroides, and *E coli*. The profound effect of C-section on the newborn's microbiota by preventing initial colonization by maternal microorganisms has been well established, whereas the strong effect of birth order on the developing microbiota is far less recognized. A few previous studies indicated that older siblings influence the infants' microbiota,[7,13,17] yet effects were not as pronounced as in our study. Furthermore, we report for the first time a dose-response relationship between birth order and microbiota composition, which lends further support to a causal relationship.

The only association between gut microbiota composition and the risk of AD development was the positive association between *Clostridium* cluster I prevalence (at ages 5 and 13 weeks) and AD.

Although several previous studies also indicated clostridia to be positively associated with AD and atopic sensitization risk,[18-20] the opposite[21,22] or no association has been reported as well.[5] Large methodological differences both about the study design as well as the microbiological techniques limit the informative value of comparing results between these studies. Most studies, including ours, only target a selected number of bacterial groups or species. As a consequence, it cannot be ruled out that reported associations merely reflect other unmeasured shifts in the microbiota composition.

A major strength of our study is the significant mediation of *Clostridium* cluster I in the association of both birth mode, confirming our previous study,[23] and birth order and AD. This further supports a causal role for the microbiota and provides evidence on the potential biological pathways by which birth mode and birth order can affect AD risk.

The present study bears several limitations that should be acknowledged. First, the study includes only children with increased risk of AD (with a positive family history of atopy). As such, the results may not be generalizable to the general population. Second, numerous associations have been tested, which can result in false-positive findings. The consistency of our results when considering different follow-up periods for both microbiota composition and AD development, but also the consistency of effects in both treatment arms of the initial intervention study (internal validation) and the supportive findings of the mediation analyses, make it unlikely that the main associations reported in this study are due to spurious findings. Third, although all C-section deliveries in the present study were preplanned, we had no information on the indication for the C-section (eg, obstetric history, anthropometric measures).

The microbiota composition was not associated with sensitization in our study. This does, in our opinion, however, not rule out that the mechanism by which the microbiota might influence AD is IgE-mediated. A large proportion of sensitized children did not show signs of AD in the present study, a finding that is consistent with previous studies. It has previously been shown that specific definitions of sensitization, such as persistent sensitization over a longer period, polysensitization, or high-level sensitization, correlate better with AD.[24] As such, the lack of association between fecal microbiota and sensitization in the present study might be due to a large number of transient "sensitized" infants with temporary IgE production who will not develop AD.

Targeted approaches, such as quantitative PCR-based methods, might seem outdated, given the introduction of next-generation sequencing approaches that enable characterization of the entire microbiome in great depth. Yet, although such techniques have instigated research in the field of the gut microbiome and have already been successfully applied in studies that relate the microbiome to numerous diseases, including allergies,[6,22] the relatively high costs of these techniques and the complex data analyses still limit their application in large-scale longitudinal

studies. Because such studies are a necessity to take numerous determinants of the microbiota together with potential confounding variables into account, molecular methods that target indicator bacteria are still indispensable.

At present, feces is the only realistic sample in large noninvasive epidemiologic studies on the gut microbiota. However, a limitation of using fecal samples is that the bacterial composition in the lumen does not necessarily reflect the composition of bacteria adhering to the mucosal surfaces.

The strong association between gut microbiota composition and number of older siblings is of special interest, given the long-recognized inverse association between birth order and the risk of allergies.[25] Identifying the mechanisms underlying this sibling or birth order effect is of great value, because it could help to develop novel tools or approaches to possibly prevent as much as 30% of all atopy cases.[26] Next to the original explanation of exposure to pathogens from older siblings in the family, alternative explanations such as *in utero* programming and endocrine effects have been postulated. Our results are in favor of the gut microbiota composition as an (additional) mechanism underlying this birth order effect, because we found the microbiota to vary with sibship size as well as to be associated with AD risk. In particular, colonization by *Clostridium* cluster I appeared to be more frequent in children with a lower birth order and to be associated with an increased risk of developing AD, which resulted in significant mediation of the association between birth order and AD risk.

We conclude from our results that the indigenous microbiota composition is likely to be one of the underlying mechanisms explaining the birth order effect in the cause of allergies. Future research is especially needed to understand how infants sample their environment over time (eg, whether, when, and to what extent exchange of microorganisms from older siblings to newborns contributes to the establishment of their microbiome), and if this relates to the risk of developing allergies and atopic manifestations such as AD.

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Key messages

- ***Clostridium* cluster I colonization in neonates is associated with an increased risk of atopic dermatitis.**
- **Next to birth mode and breast-feeding, birth order is a strong determinant of the infant microbiota composition.**

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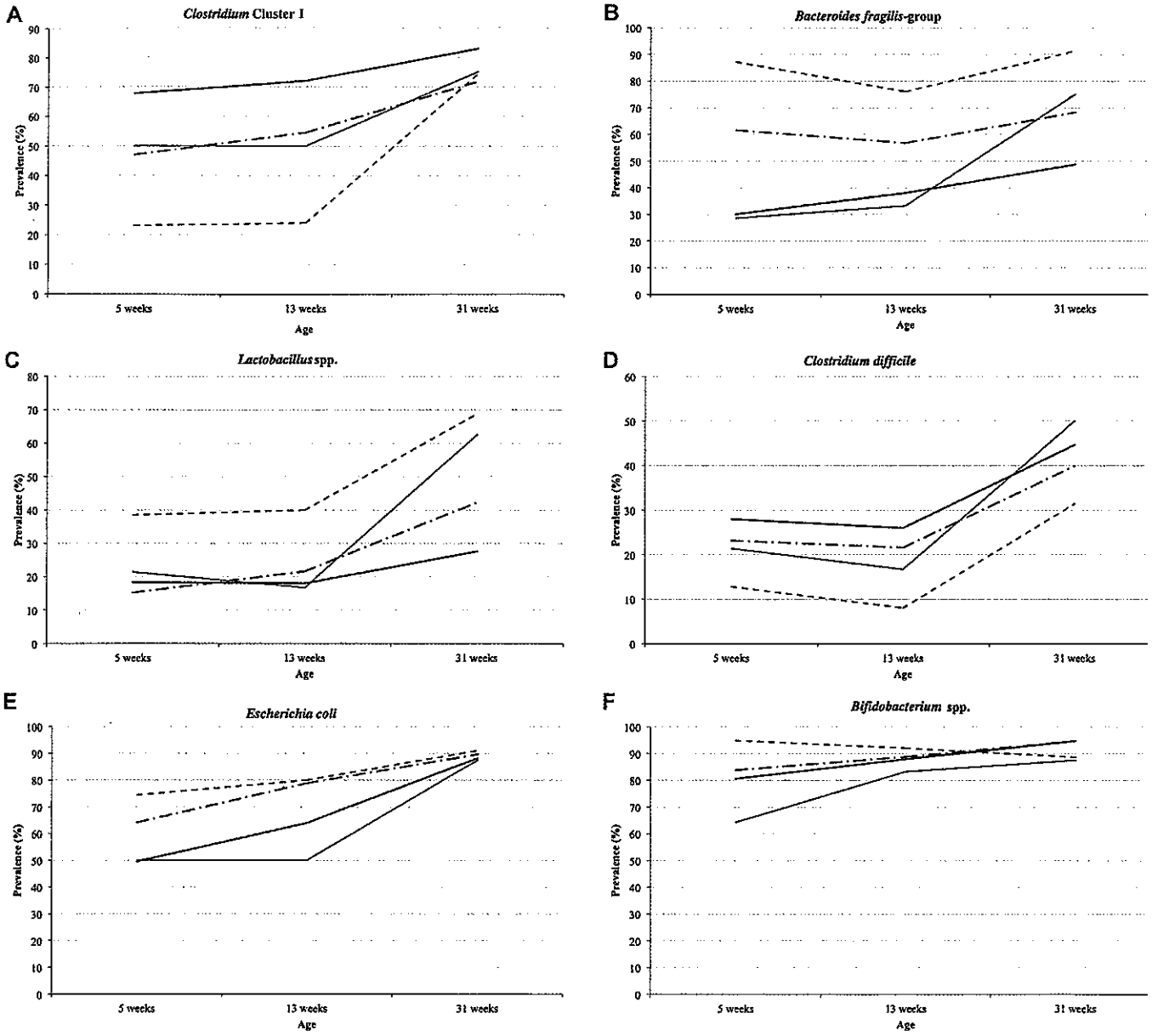

FIG E1. Colonization pattern of gut bacteria during the first 31 weeks of life, presented as the proportion of infants colonized at each time point according to mode of delivery and sibling status (*dark gray solid line*, no siblings and C-section delivery; *dark gray dashed line*, no siblings and vaginal delivery; *light gray solid line*, ≥2 siblings and C-section delivery; *light gray dashed line*, ≥2 siblings and vaginal delivery) for *Clostridium* Cluster I **(A)**, *Bacteroides fragilis* group **(B)**, *Lactobacillius* spp **(C)**, *Clostridium difficile* **(D)**, *Escherichia coli* **(E)**, and *Bifidobacterium* spp **(F)**.

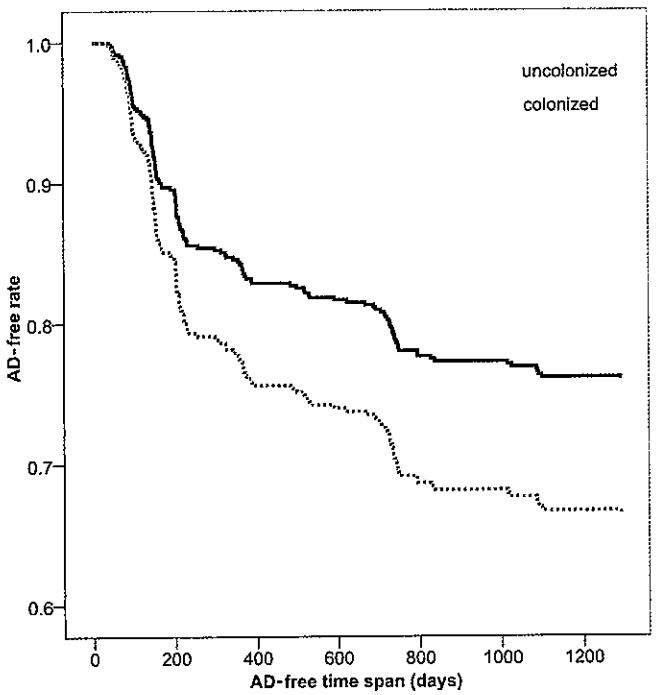

FIG E2. Survival plot for the development of AD in children colonized and noncolonized by *Clostridium* cluster I at age 5 weeks. Derived from Cox regression analyses adjusted for sex, birth weight, maternal and paternal atopy, (duration of) breast-feeding, number of siblings, birth mode, treatment group, and prevalence of the other bacteria under study. Adjusted hazard ratio = 1.49; 95% CI, 1.05-2.11; $P = .03$.

TABLE E1. Primers and probes used in this study

Target organisms	Primer/probe	Sequence (5′-3′)	T_m (°C)	Reference
Bifidobacterium spp	Forward primer	GCGTGCTTAACACATGCAAGTC	59	E1
	Reverse primer	CACCCGTTTCCAGGAGCTATT	59	E1
	Probe	TCACGCATTACTCACCCGTTCGCC	70	E1
E coli	Forward primer	CATGCCGCGTGTATGAAGAA	59	E2
	Reverse primer	CGGGTAACGTCAATGAGCAAA	59	E2
	Probe	TATTAACTTTACTCCCTTCCTCCCCGCTGAA	68	E2
C difficile	Forward primer	TTGAGCGATTTACTTCGGTAAAGA	58	E1
	Reverse primer	TGTACTGGCTCACCTTTGATATTCA	59	E1
	Probe	CCACGCGTTACTCACCCGTCCG	69	E1
B fragilis group	Forward primer	CGGAGGATCCGAGCGTTA	58	E1
	Reverse primer	CCGCAAACTTTCACAACTGACTTA	59	E3
	Probe	CGCTCCCTTTAAACCCAATAAATCCGG	68	E1
Lactobacillus spp	Forward primer	AGCAGTAGGGAATCTTCCA	59	E4,E5
	Reverse primer	CACCGCTACACATGGAG	59	E4,E6
Clostridium cluster I	Forward primer	TACCHRAGGAGGAAGCCAC	55	E7
	Reverse primer	GTTCTTCCTAATCTCTACGCAT	53	E7
	Probe	GTGCCAGCAGCCGCGGTAATACG	72	E7

T_m, Melting temperature.

TABLE E2. Adjusted association for mode of delivery and sibship size in relation to the risk of colonization with the bacteria under study at ages 5, 13, and 31 weeks

	Mode of delivery*		Number of siblings		
	Spontaneous†	C-section	0†	1	≥2
Age 5 wk					
Clostridium cluster I	1.0	3.05 (2.03-4.58)‡	1.0	0.56 (0.38-0.81)‡	0.33 (0.17-0.63)‡
Bacteroides	1.0	0.22 (0.14-0.36)‡	1.0	1.00 (0.68-1.46)	2.68 (1.32-5.43)‡
Bifidobacteria	1.0	0.59 (0.33-1.05)	1.0	3.77 (1.78-7.93)‡	1.08 (0.46-2.57)
C difficile	1.0	1.85 (1.20-2.86)‡	1.0	0.90 (0.59-1.38)	0.55 (0.25-1.24)
E coli	1.0	0.47 (0.32-0.70)‡	1.0	1.24 (0.85-1.80)	1.51 (0.80-2.85)
Lactobacilli	1.0	0.91 (0.56-1.39)	1.0	1.67 (1.07-2.60)‡	2.26 (1.16-4.40)‡
Age 13 wk					
Clostridium cluster I	1.0	1.99 (1.17-3.36)‡	1.0	0.54 (0.33-0.88)‡	0.28 (0.12-0.64)‡
Bacteroides	1.0	0.40 (0.24-0.68)‡	1.0	1.15 (0.70-1.88)	1.96 (0.87-4.42)
Bifidobacteria	1.0	0.90 (0.40-2.05)	1.0	1.53 (0.65-3.57)	1.25 (0.35-4.45)
C difficile	1.0	1.97 (1.12-3.48)‡	1.0	1.20 (0.69-2.11)	0.36 (0.10-1.23)
E coli	1.0	0.51 (0.29-0.90)	1.0	1.51 (0.83-2.75)	0.99 (0.41-2.36)
Lactobacilli	1.0	1.17 (0.66-2.07)	1.0	2.17 (1.27-3.71)‡	1.99 (0.89-4.48)
Age 31 wk					
Clostridium cluster I	1.0	1.56 (0.93-2.61)	1.0	0.90 (0.58-1.40)	0.95 (0.46-1.95)
Bacteroides	1.0	0.43 (0.28-0.68)‡	1.0	1.58 (1.04-2.42)‡	4.61 (1.75-12.16)‡
Bifidobacteria	1.0	1.04 (0.43-2.51)	1.0	0.85 (0.37-1.93)	0.44 (0.15-1.30)
C difficile	1.0	1.26 (0.82-1.92)	1.0	1.11 (0.75-1.64)	0.82 (0.43-1.64)
E coli	1.0	0.77 (0.39-1.52)	1.0	1.36 (0.71-2.62)	1.81 (0.53-6.23)
Lactobacilli	1.0	0.68 (0.44-1.04)	1.0	1.65 (1.11-2.46)‡	2.63 (1.36-5.10)‡

*Values are odds ratios and 95% CIs from logistic regression analyses on mode of delivery and sibship size in association to colonization by the bacteria under study.
†Reference category.
‡$P < .05$.

TABLE E3. Effects of environment/diet on GI microbiota at the ages of 5, 13, and 31 weeks

	Bifidobacteria	Lactobacilli	*C difficile*	*Clostridium* cluster I	Bacteroides	*E coli*
Low birth weight						
5 wk	Decrease	—	—	—	Decrease	—
13 wk	—	—	Increase	—	—	—
31 wk	—	—	Increase	—	—	—
Sex						
5 wk	—	—	—	—	—	—
13 wk	—	—	—	—	—	—
31 wk	—	—	—	—	—	—
C-section						
5 wk	Decrease	—	Increase	Increase	Decrease	Decrease
13 wk	—	—	Increase	Increase	Decrease	—
31 wk	—	Decrease	—	Increase	Decrease	—
Siblings						
5 wk	Increase	Increase	Decrease	Decrease	Increase	—
13 wk	—	Increase	—	Decrease	—	—
31 wk	—	Increase	—	—	Increase	Increase
Duration of breast-feeding						
31 wk	Increase	Increase	Decrease	—	Decrease	—
Day care						
31 wk	—	—	—	—	—	—
Atopy father						
5 wk	—	—	—	—	—	—
13 wk	—	—	—	—	—	—
31 wk	—	—	—	—	—	—
Atopy mother						
5 wk	—	—	—	—	—	—
13 wk	—	—	—	—	—	—
31 wk	—	—	—	—	—	—

No effect was seen for sex, day care, atopy of father, and atopy of mother.
—, No effect seen.

TABLE E4. Univariable associations between treatment group and median counts and prevalence of colonization of selected gut bacteria in feces of children at age 31 weeks

Treatment group	No.	*Bifidobacterium* spp, counts* (%)	*Lactobacillus* spp, counts* (%)	*C difficile*, counts* (%)	*E coli*, counts* (%)	*Clostridium* cluster I, counts* (%)	*B fragilis* group, counts* (%)
Active	245	9.04 (93.9)	6.29 (48.2)	7.03 (41.6)	8.83 (89.4)	5.41 (74.7)	10.09 (70.2)
Placebo	254	9.04 (93.7)	6.32 (49.4)	6.77 (40.2)	8.72 (89.8)	5.52 (72.8)	10.14 (66.1)
P value†		.59	.76	.58	.29	.55	.23

*Counts are presented as median values (log_{10} CFU/mL feces) calculated from positive samples only.
†Determined by Mann–Whitney rank sum test calculated from all samples (the statistical significance refers to on overall difference incorporating both counts and prevalence).

TABLE E5. Adjusted associations between colonization with the gut bacteria at age 13 weeks and the development of AD between age 5 and 31 weeks (n = 318)

	Atopic dermatitis	
	OR (95% CI)	$OR_{adjusted}$ (95%CI)*
Bifidobacterium spp		
No†	1.0	1.0
Yes	1.2 (0.47-3.04)	1.72 (1.64-4.63)
E coli		
No†	1.0	1.0
Yes	1.24 (0.65-2.40)	0.93 (0.45-1.92)
C difficile		
No†	1.0	1.0
Yes	1.12 (0.59-2.12)	1.13 (0.55-2.32)
B fragilis group		
No†	1.0	1.0
Yes	0.72 (0.42-1.24)	0.67 (0.41-1.39)
Lactobacillus spp		
No†	1.0	1.0
Yes	0.69 (0.37-1.31)	0.55 (0.27-1.14)
Clostridium cluster I		
No†	1.0	1.0
Yes	1.67 (0.96-2.89)	2.51 (1.30-4.86)‡

*Analyses were adjusted for sex, birth weight, maternal and paternal atopy, (duration of) breast-feeding, number of siblings, mode of delivery, and treatment group.
†Reference category.
‡$P < .05$.