Gut microbiota and development of atopic eczema in 3 European birth cohorts

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Background: Stimulation of the immune system by gut microbes might prevent allergy development.
Objective: The present study examined the hypothesis that sensitization to food allergens and atopic eczema are influenced by the infantile intestinal colonization pattern.
Methods: Infants were recruited perinatally in Göteborg (n = 116), London (n = 108), and Rome (n = 100). Commensal bacteria were identified to the genus or species level in rectal (3 days) and quantitative stool cultures (7, 14, and 28 days and 2, 6, and 12 months of age). At 18 months of age, atopic eczema and total and food-specific IgE levels were assessed. These outcomes were modeled in relation to time to colonization with 11 bacterial groups and to ratios of strict anaerobic to facultative anaerobic bacteria and gram-positive to gram-negative bacteria at certain time points. Study center, mode of delivery, parity, and infant diet were included as covariates.
Results: Neither atopic eczema nor food-specific IgE by 18 months of age were associated with time of acquisition of any particular bacterial group. Cesarean section delayed colonization by *Escherichia coli* and *Bacteroides* and *Bifidobacterium* species, giving way to, for example, *Clostridium* species. Lack of older siblings was associated with earlier colonization by *Clostridium* species and lower strict anaerobic/facultative anaerobic ratio at 12 months.
Conclusions: This study does not support the hypothesis that sensitization to foods or atopic eczema in European infants in early life is associated with lack of any particular culturable intestinal commensal bacteria.
Clinical implications: The nature of the microbial stimulus required for protection from allergy remains to be identified.
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Key words: *Allergy, atopic eczema, sensitization, IgE, intestine, commensal bacteria, infant*

The causes of the ''allergy epidemic'' in Western countries[1] are unknown. The hygiene hypothesis postulates that deprivation of microbial exposure in infancy predisposes to immune dysregulation and allergy development.[2,3] This is based on observations that children growing up in poor[3] or large[3] families or with close animal contacts[4,5] have reduced risk of allergy. Childhood infections are associated with reduced allergy development, especially gastrointestinal infections,[6-8] but no particular protective microbial stimuli have yet been identified.

The commensal intestinal microbiota could play a role in shaping the developing immune system and in protecting against allergy development. Acquisition of the commensal microbiota proceeds in a sequential manner, with facultative (oxygen-tolerant) anaerobic bacteria establishing early. Strict anaerobic bacteria establish successively until an adult-type microbiota dominated by strict anaerobic bacteria by a factor of 1000:1 is established.[9] Infants in industrialized countries are colonized later than those in developing countries by fecal bacteria[10] and have slow strain turnover in their microbiota.[11] This could lead to a lesser stimulation of the immune systems of westernized infants because intestinal bacteria evoke an immune response transiently on their establishment in the microbiota and further persistence of the same strain does not further stimulate immunity.[12] Development of immunologic tolerance to dietary antigens, which occurs normally in human subjects and animals,[13,14] is, at least in part, dependent on the presence of commensal microbes.[15] Because gram-positive and gram-negative bacteria affect antigen-presenting cells differently,[16] the composition of the microbiota could influence the immune response to innocuous environmental antigens.

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It has been hypothesized that the ''Western microbiota'' fails to support maturation of immunologic tolerance to environmental antigens because of the disappearance of key groups of microorganisms necessary for tolerance development or because of a secondary expansion of groups of bacteria that hamper the induction of tolerance.[17] Indeed, certain studies have reported differences in the early intestinal microbiota between infants developing and those not developing allergic disease, with more prominent colonization by *Bifidobacterium* species but less by *Clostridium* species in the latter group.[18-20] The results are, however, not univocal, and a recent large study saw no protective effect of bifidobacteria but reported that colonization by *Clostridium difficile* at 1 month of age was associated with later allergy development.[21]

In the present birth cohort study, including more than 300 infants, we investigated whether colonization by culturable fecal bacteria is related to the development of atopic eczema and sensitization to at least 1 of the most common food allergens by 18 months of age in Swedish, British, and Italian infants, taking into account the possible influence of lifestyle and dietary factors. This is, to date, the largest cohort followed longitudinally with respect to establishment of the intestinal microbiota. Furthermore, inclusion of infantile populations from 3 European countries increases the generalizability of the findings.

METHODS

Study design

We recruited newborn infants at Sahlgrenska University Hospital, Göteborg; St George's Hospital, London; and Sandro Pertini Hospital, Rome. A normal singleton pregnancy was the only inclusion criterion, whereas language difficulty, moving house soon, or prematurity (<37 weeks' gestation) were exclusion criteria. Atopic heredity, family structure, and exposure to animals were ascertained by questionnaire, and information on delivery and antibiotic treatment of the mother or baby was retrieved from hospital records. Parental interviews at 6 and 12 months of age probed for feeding pattern, infections, and medication. Maternal diet while breastfeeding was not considered.

At 18 months, all infants were examined through a standardized questionnaire and physical examination. Atopic eczema was diagnosed according to Williams' criteria,[22] and a SCORAD value was obtained by using validated software (SCORAD-Card; TPS Production, Rome, Italy).[23] Serum total IgE (IgE-FEIA; Pharmacia Diagnostics, Uppsala, Sweden) and specific IgE levels against common food antigens (FX5 food mix: egg white, cow's milk, fish, peanut, soya, and wheat; Immunocap FEIA, Pharmacia Diagnostics) were tested centrally.

Informed consent was obtained, and the study was approved by local ethics committees.

Culture and identification of bacteria in intestinal microbiota

A rectal swab was collected at 3 days of age and cultured semiquantitatively for facultative anaerobic bacteria within 24 hours, as described in detail elsewhere.[24] Fecal samples were collected at 7, 14, and 28 days and 2, 6, and 12 months of age and transported under anaerobic conditions to the laboratory, where serial dilutions were plated on selective media and cultured aerobically and anaerobically within 24 hours.[24] Free-lying colonies of different morphology were separately enumerated and subcultured for purity, including confirmation of inability to grow aerobically for strict anaerobes (except for gram-positive rods, for which scanty aerobic growth was accepted).[24] The limit of detection was 330 ($10^{2.52}$) colony-forming units/g of feces. Total strict anaerobic and facultative anaerobic bacterial counts were calculated from growth on nonselective media incubated anaerobically and aerobically, respectively.[24]

Bacteria were identified through Gram staining and biochemical/genetic tests.[24] *Enterobacteriaceae* were isolated from Drigalski agar and speciated with API20E (API Systems, Montalieu-Vercieu, France). *Staphylococcus aureus* and coagulase-negative staphylococci were isolated from *Staphylococcus* agar and identified by using catalase and coagulase tests. Enterococci were identified by means of esculine hydrolysis on Enterococcosel agar. *Bacteroides* species were isolated from *Bacteroides* Bile Esculin agar and speciated by using Rapid ID 32A (API Systems). *Clostridium* species were defined as straight gram-positive or gram-labile rods isolated from alcohol-treated samples (which kills vegetative cells but leaves spores intact), cultivated on Brucella blood agar, and speciated with Rapid ID 32A. *Bifidobacterium* species were isolated from Beerens' agar and identified by using genus-specific PCR.[25] *Lactobacillus* species were isolated from Rogosa agar and identified by means of PCR with species-specific primers.[26] Total gram-positive and gram-negative bacterial counts were calculated by combining the counts of each gram-positive and gram-negative bacterial group, respectively.

All centers used identical methods and reagents, and staff were trained centrally. Uniformity in analysis was checked repeatedly by means of blinded analysis of the same fecal sample in the 3 laboratories.

Data handling and statistics

Databases were transferred to STATA version 8.2 (Stata Corp, College Station, Tex) for linkage, cleaning, and statistical analysis.

For each major bacterial group, the presence or absence in the feces at each time point was determined. The cumulative incidence of colonization was derived by combining the data across the 7 time points (6 for strict anaerobic bacteria, which were not cultured at 3 days of age). The ratios of strict anaerobic to facultative anaerobic counts and gram-negative to gram-positive counts were evaluated at each time point.

Regression models were constructed by using time to colonization for each bacterial group as an ordinal 7- or 8-category explanatory variable (including never colonized) or log-transformed strict anaerobic/facultative anaerobic ratio or gram-negative/gram-positive ratio at specific time points as a continuous explanatory variable. Atopic eczema at 18 months of age was analyzed as a dichotomous outcome variable, as were food-specific IgE (no vs any positive reaction) and total IgE levels (dichotomized at 100 kU/L). Total IgE was also analyzed as a continuous variable after logarithmic transformation. Recruitment center was included as a 3-level covariate in all analyses. In a second model, mode of delivery, parity, and breast-feeding at 6 months were included as 2-level covariates. In a third model, timing of introduction of certain solid foods was introduced as a continuous covariate.

Results are presented as *P* values and direction of association (rather than estimates of magnitude of effect) because of the variety of exposure metrics and our preference for test for linear trend across all exposure categories rather than specific 2-group comparisons. Acknowledging the large number of interrelated hypotheses tested, only *P* values of .01 or less were considered significant.

TABLE I. Population, risk/protective factors, and allergic outcomes

	Prevalence (%)			
	Göteborg (116 children)	London (108 children)	Rome (100 children)	All centers (324 children)
Male sex	49	60	49	53
Maternal history of allergy	68	51	29	50
Paternal history of allergy	61	48	37	49
Parental history of allergy	88	72	56	73
Vaginal delivery	85	70	61	73
Antibiotics during pregnancy	6	36	51	30
Siblings (≥1)	46	47	46	46
Antibiotics to child by 6 mo	8	36	29	24
Pets in the house at 6 mo	15	19	25	19
Nonparental caregiver at 6 mo	5	22	15	14
Exclusive breast-feeding at 6 mo	24	12	37	24
Total IgE >25 kU/L	28	33	49	36
Total IgE >100 kU/L	5	15	17	12
Food-specific IgE	35	20	22	26
Atopic eczema	29	33	6	23

RESULTS

Allergy outcomes and prevalence of risk factors in the 3 centers

Atopic eczema affected approximately one third of Swedish and British infants but only 6% of Italian infants. Food-specific IgE antibodies were common in all 3 cohorts. Increased total serum IgE levels (>100 kU/L) were observed in one sixth of the children in Rome and London but only in 5% of Swedish children (Table I).

Factors traditionally regarded as associated with allergy development were not uniformly distributed among the 3 cohorts; for example, parental allergy was very common in the Swedish and British children, whereas cesarean section was most common in the Italian cohort (Table I).

Intestinal colonization pattern

Intestinal colonization of the combined cohort by each of the major bacterial groups is shown in Fig 1. Among facultative anaerobic bacteria, coagulase-negative staphylococci were the earliest colonizers, followed by enterococci. *Escherichia coli* and other *Enterobacteriaceae*, traditionally recognized as early colonizers, appeared late, and not until 6 months of age had *E coli* been established in practically all infants. *S aureus* was almost as common as *E coli* in the microbiota during the first 2 months of life (Fig 1, *A*). Among anaerobic bacteria, bifidobacteria were the earliest colonizers, followed by *Clostridium* and *Bacteroides* species. Persistent colonization by lactobacilli was rare (data not shown), but the majority were at least transiently colonized during their first year of life (Fig 1, *C*).

The stool population counts of different bacterial groups in colonized infants were calculated. Facultative anaerobic bacteria, especially staphylococci, decreased in population counts with time (Fig 1, *B*). In contrast, most strict anaerobic bacteria maintained their population levels in the increasingly complex microbiota (Fig 1, *D*). The ratio of the geometric means for strict to facultative anaerobic bacterial counts was 0.10 at 7 days, 4.4 at 2 months, and 60 at 12 months of age. Gram-positive bacteria dominated the early microbiota, but the counts of gram-negative bacteria increased with time: the ratio of the geometric means for gram-negative to gram-positive bacterial counts was 0.004 at 7 days, 0.09 at 2 months, and 0.32 at 12 months of age.

Colonization patterns in relation to lifestyle factors

We tested whether lifestyle factors proposed as linked to allergy were related to the intestinal colonization pattern. Several highly significant associations could be observed (Table II). In all 3 centers, infants delivered by means of cesarean section were colonized later and less frequently by *Bifidobacterium* species, *Bacteroides* species, and *E coli*. Instead, *Clostridium* species and Enterobacteriaceae other than *E coli* were more prevalent after cesarean section, and the ratio of strict to facultative anaerobes was decreased in these infants (Table II). Similar to the cesarean section–delivered infants, first-born children were more rapidly colonized by *Clostridium* species and by Enterobacteriaceae other than *E coli* and had lower strict/facultative anaerobic ratio at 12 months of age. These associations persisted after exclusion of children delivered by means of cesarean section (Table II).

Postponement of introduction of different solids and longer duration of breast-feeding was associated with delayed colonization by *Klebsiella* species and also often with a reduced strict/facultative anaerobic ratio (Table II). Antibiotic treatment of the mother or child was associated with a decreased ratio of strict to facultative anaerobes (Table II).

Atopy and atopic eczema in relation to colonization patterns

The presence of atopic eczema or food-specific IgE by 18 months of age was modeled as a function of time to

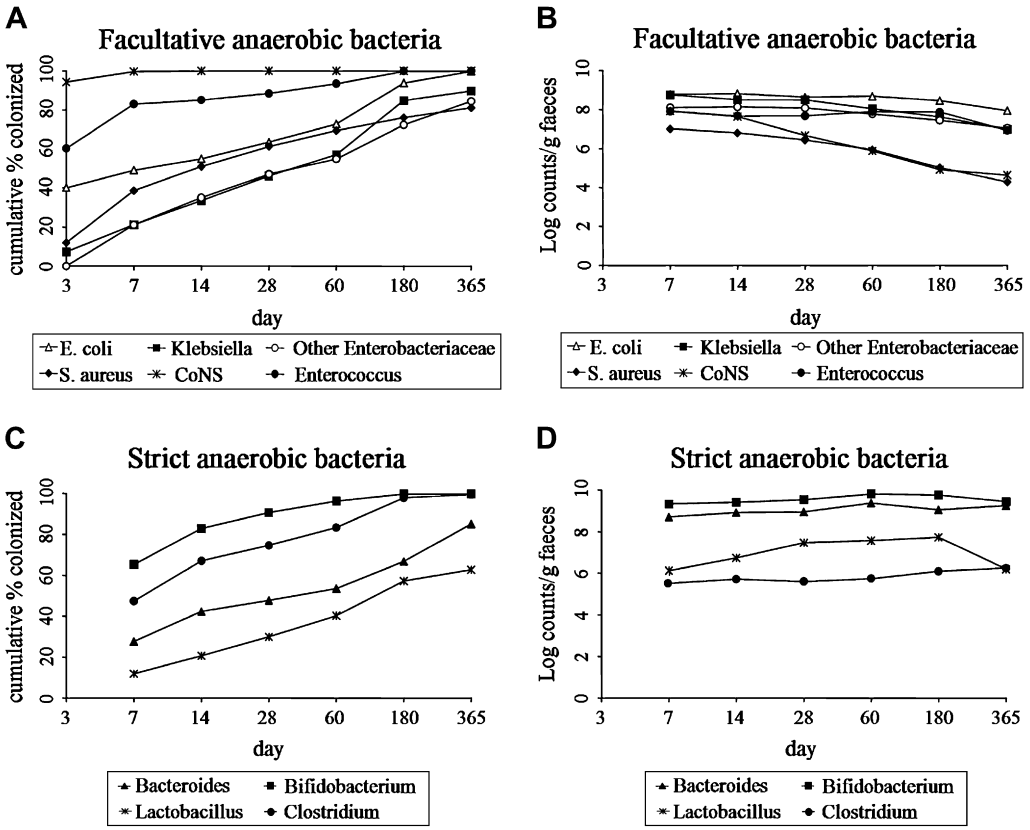

FIG 1. Intestinal colonization pattern during the first year of life. The results are presented as the proportion of infants ever colonized by each time point and the mean $\log_{10}$ count for colonized infants only at each time point for facultative anaerobic bacteria (**A** and **B**) and strict anaerobic bacteria (**C** and **D**). *CoNS,* Coagulase-negative staphylococci.

colonization for each bacterial group or to strict anaerobes/facultative anaerobes or gram-positive/gram-negative ratios at 3 time points. All analyses were adjusted for center. No statistically significant associations were found (Table III). Adjustment for mode of delivery, parity, and breast-feeding at 6 months only marginally affected the results, and no significant associations emerged (data not shown). The same was true if each bacterial variable was adjusted for the particular confounders that were significantly associated with the bacterial group in question (Table II). Higher gram-negative/gram-positive ratios at 12 months of age were associated with IgE levels of greater than 100 kU/L at 18 months (P = .007, Table III). Adjustment for confounders did not alter this association (data not shown).

When center-adjusted, log-transformed total IgE was analyzed as a continuous outcome variable (offering greater statistical power), later colonization with *Lactobacillus* species emerged as a borderline significant association (P = .03, Table III). The association remained after adjustment for mode of delivery, parity, and breast-feeding for 6 months (P = .02) and after further adjustment for dietary correlates (P = .03). The relation between colonization by lactobacilli and the 3 outcome variables of atopic eczema, food-specific IgE, and total IgE is shown in Fig 2. For comparison, colonization by bifidobacteria is shown in the same panels.

DISCUSSION

This is the largest and most comprehensive study to date of the intestinal colonization pattern in infancy. Meticulous calibration of methodology permitted comparisons across centers.

Using this culture-based approach, we could demonstrate slow acquisition of typical fecal bacteria, such as *E coli*, compared with studies from the last 30 years,[9] suggesting a reduced circulation of fecal bacteria in contemporary Western societies. Reduced competition from traditional intestinal microbes might have paved the way for the frequent colonization by staphylococci noted here and reported previously.[24] A third sign of a hygiene-associated colonization pattern was the quite slow decrease in facultative anaerobic bacterial population levels in response to competition from an increasingly complex microbiota.[9] The results clearly demonstrate that a hygienic lifestyle not only affects the incidence of

TABLE II. Associations between demographic/lifestyle factors and intestinal colonization pattern

Demographic/ lifestyle factor	Acquisition of defined bacterial groups				Bacterial population ratios					
	Earlier		Later		Strict/facultative anaerobes			Gram-negative/ gram-positive		
	Species/genus	*P* value	Species/genus	*P* value	Time	Effect	*P* value	Time	Effect	*P* value
Cesarean delivery	*Klebsiella* species	.003	*Escherichia coli*	<.001	7 d	↓	<.001	7 d	↓	.025
	Other *Enterobactericeae**	<.001	*Bacteroides* species	<.001	12 mo	↓	.040			
	Clostridium species	<.001	*Bifidobacterium* species	<.001						
Parental allergy								2 mo	↓	.048
Pets in house at 6 mo of age										
First child—all deliveries	Other *Enterobactericeae*	.010			12 mo	↓	<.001			
	Clostridium species	<.001								
First child—vaginal deliveries	*Klebsiella* species	.010			12 mo	↓	.001	2 mo	↑	.041
	Other *Enterobactericeae*	.020								
	Clostridium species	<.001								
Male sex								12 mo	↓	.009
Nonparental caregiver 6 mo			*Bifidobacterium* species	.030						
Antibiotics during pregnancy			*Bacteroides* species	.040	12 mo	↓	.003			
Antibiotics to child first 6 mo			*Clostridium* species	.030	2 mo	↓	.002			
Exclusive breast-feeding at 6 mo	*Escherichia coli*	.030	*Klebsiella* species	.030				2 mo	↓	.048
Longer duration of breast-feeding	*Bifidobacterium* species	.050	*Klebsiella* species	.006						
			Clostridium species	.015						
Later introduction of cow's milk			*Klebsiella* species	.003						
Later introduction of dairy products			*Staphylococcus aureus*	.040	2 mo	↓	.038			
Later introduction of bread	*Escherichia coli*	.040								
	Lactobacillus species	.020								
Later introduction of porridge			CoNS	.030	12 mo	↑	.049			
Later introduction of eggs			*Klebsiella* species	.004	2 mo	↓	.036			
			Bacteroides species	.040						
Later introduction of potatoes			*Klebsiella* species	.040						
Later introduction of vegetables			*Klebsiella* species	.005	7 d	↓	.021			
Later introduction of fruit					7 d	↓	.013			

Only associations with the studied microbial variables at a *P* value of less than .05 are shown.
CoNS, coagulase-negative staphylococci.
**Enterobactericeae* other than *Escherichia coli* or *Klebsiella* species.

childhood infections[2] but also profoundly influences the intestinal colonization pattern.

Furthermore, significant associations between intestinal colonization pattern and several medical or lifestyle factors were readily demonstrated. Thus delivery by means of cesarean section, which deprives the baby of contact with the maternal vaginal, perineal, and fecal flora, was associated with delayed acquisition of *E coli*, bifidobacteria, and *Bacteroides* species and a compensatory increase in colonization by *Clostridium* and *Klebsiella* species and other non–*E coli* enterobacteria. These findings are in accordance with previous studies.[24,27] *E coli* is confined to the bowel microbiota of human subjects and animals, and its presence signifies fecal contamination. If *E coli* is not passed from mother to infant, which occurs in approximately one third of vaginal deliveries,[9] acquisition of this species is difficult. In contrast, other Enterobacteriaceae, such as *Klebsiella* and *Enterobacter* species, are also found in the environment, including in foods.[28] In accordance, early introduction of several solids was associated with accelerated colonization with certain bacteria, mostly *Klebsiella* species. *E coli* is a superior gut colonizer, but in its absence *Klebsiella* species or other Enterobacteriaceae might expand. Clostridia form spores

TABLE III. Associations between allergic outcomes at age 18 months and earlier colonization with each bacterial group and ratios of bacterial counts at 7 days, 2 months, and 12 months, adjusted for center

Bacterial group or exposure index	Atopic eczema		Food-specific IgE		Total IgE >100 kU/L		Total IgE* (continuous)	
	Risk	*P* value†	Risk	*P* value	Risk	*P* value	Level	*P* value
Earlier colonization with:								
All Enterobacteriaceae	↑		↓		↑		↑	
Escherichia coli	↑		↓		↑		↑	
Klebsiella species	↓		↑		↑		↑	
Other *Enterobacteriaceae*	↓		↑		–		↓	
Coagulase-negative staphylococci	↓		↑		–		↑	.15
Staphylococcus aureus	↑	.06	–		↑		↑	
Enterococcus species	↓		–		↑		↑	
Bacteroides species	↑	.06	↑	.11	↑		↑	
Bifidobacterium species	↓		↓		↑		↑	
Lactobacillus species	↓		↓	.09	↓		↓	.03
Clostridium species	↑		↑		↑		↑	
Higher strict anaerobic/facultative anaerobic ratio								
7 d	↑	.11	↑		↑		↑	
2 mo	↑		↓	.14	↓		↓	
12 mo	↑		↓	.07	↓		↑	
Higher gram-negative/gram-positive ratio								
7 d	↓		↓		↑		↓	
2 mo	↑		↓		↓	.07	↓	.13
12 mo	↓		↑		↑	.007	↑	.03

↑, Higher risk of being affected by a specific atopic outcome/higher level of total IgE; ↓, lower risk of being affected by a specific atopic outcome/lower level of total IgE.

*Modeled as a log-transformed continuous outcome variable.

†Only *P* values of .15 or less are shown.

that resist disinfectants, desiccation, and heat and are ubiquitous, even in highly hygienic environments. They might thus replace other anaerobes, such as *Bacteroides* species, an anaerobe that has difficulties in spreading under good sanitary conditions. Furthermore, the strict/facultative anaerobe ratio was strongly decreased initially and remained low, even at 12 months of age, in infants delivered by means of cesarean section, as evidence of a delayed maturation of the intestinal microbiota. Being first born was also associated with increased prevalence of opportunistic colonizers, such as clostridia and Enterobacteriaceae other than *E coli* and a relatively weaker dominance of anaerobes in the microbiota at 1 year of age. This indicates quite profound and hitherto unreported effects on the commensal microbiota by sibship position, a variable strongly linked to allergy.[3] The colonization pattern of children without siblings thus resembled that of infants born by means of cesarean section, although the observed effects were weaker. Treatment of the infant or pregnant mother with antibiotics was also associated with a delayed establishment of anaerobic dominance in the microbiota. An influence on the microbiota by perinatal antibiotic treatment has previously been reported.[24]

Despite these clear-cut findings of an effect of lifestyle on intestinal colonization pattern, we could not demonstrate any significant associations between fecal microbes and development of atopic eczema or specific IgE to food antigens. Previous associations of *Bifidobacterium* species with protection against allergy development[18-20,29,30] based on smaller infant groups were thus not confirmed. We also saw no association between the opportunistic colonizers *Clostridium* species or *C difficile* (data not shown) and enhanced risk of allergy, as previously suggested.[18-21] A borderline significant association between increased total serum IgE levels at 18 months of age and delayed colonization by *Lactobacillus* species was observed but should be interpreted with caution in view of the large number of analyses performed. Furthermore, total serum IgE levels in infancy might correlate poorly with total IgE levels later in life[31] and have low capacity to predict later sensitization to inhalant allergens.[32] The dissociation between total IgE and atopy was illustrated by the finding that increased total IgE levels were most common in the Italian cohort, which had the lowest incidence of atopic eczema.

We observed a gradual increase in the proportion of gram-negative relative to gram-positive bacteria with age because the early gram-positive anaerobic colonizers (eg, bifidobacteria and clostridia) were joined by *Bacteroides* species. Infants with increased total serum IgE levels at 18 months had significantly higher counts of gram-negative relative to gram-positive bacteria at 12 months of age. The significance of this finding remains to be confirmed in other studies. Possibly, exposure to LPS of gram-negative bacteria induces maturation of dendritic cells and might have an adjuvant effect on IgE production, as suggested from animal studies.[33] However, neither the

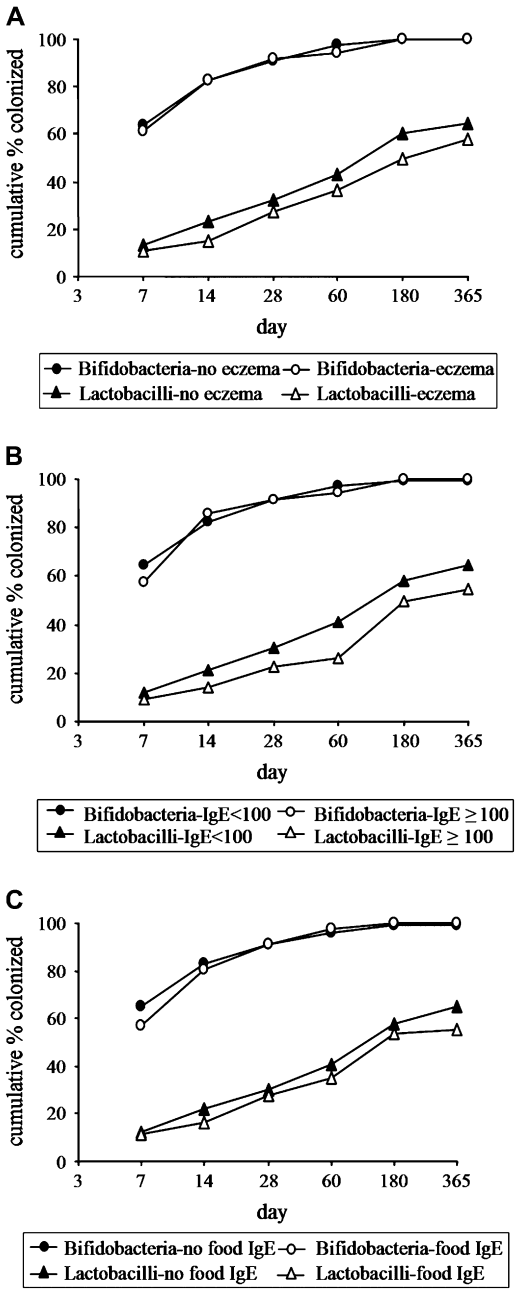

FIG 2. Colonization by *Bifidobacterium* and *Lactobacillus* species in relation to allergy outcomes. The cumulative incidence of colonization by bifidobacteria and lactobacilli in relation to atopic eczema during the first 18 months of life **(A)**, total IgE in the serum at 18 months of age **(B)**, and food-specific IgE at 18 months of age **(C)** is shown.

development of atopic eczema nor sensitization to food allergens was influenced by the ratio of gram-negative to gram-positive bacteria.

Before ruling out intestinal commensals as modulators of the risk of allergy development, the limitations of the study should be acknowledged. First, some noncultivable bacteria with strong immune-activating properties might remain undetected.[34] Second, atopic eczema was the only common clinical allergic manifestation in this group. Hay fever and asthma were very uncommon at this young age, but examination of the same cohorts at school age might reveal associations between microbiota in infancy and later development of respiratory allergy. Transient food allergy appearing and disappearing before 18 months of age was also not considered in the present study.

This combined analysis of European birth cohorts shows some conspicuous determinants of intestinal colonization patterns but does not support the hypothesis that development of IgE to food allergens or atopic eczema in infancy are associated with lack of any particular culturable intestinal commensal bacteria. The exact nature of the microbial stimulus required for normal tolerance development and protection from allergy in early infancy remains to be identified.

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