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ARTICLE

Factors Influencing the Composition of the Intestinal Microbiota in Early Infancy

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ABSTRACT

OBJECTIVE. The aim of this study was to examine the contribution of a broad range of external influences to the gut microbiotic composition in early infancy.

METHODS. Fecal samples from 1032 infants at 1 month of age, who were recruited from the KOALA Birth Cohort Study in the Netherlands, were subjected to quantitative real-time polymerase chain reaction assays for the enumeration of bifidobacteria, *Escherichia coli*, *Clostridium difficile*, *Bacteroides fragilis* group, lactobacilli, and total bacterial counts. Information on potential determinants of the gut microbiotic composition was collected with repeated questionnaires. The associations between these factors and the selected gut bacteria were analyzed with univariate and multivariate analyses.

RESULTS. Infants born through cesarean section had lower numbers of bifidobacteria and *Bacteroides*, whereas they were more often colonized with *C difficile*, compared with vaginally born infants. Exclusively formula-fed infants were more often colonized with *E coli*, *C difficile*, *Bacteroides*, and lactobacilli, compared with breastfed infants. Hospitalization and prematurity were associated with higher prevalence and counts of *C difficile*. Antibiotic use by the infant was associated with decreased numbers of bifidobacteria and *Bacteroides*. Infants with older siblings had slightly higher numbers of bifidobacteria, compared with infants without siblings.

CONCLUSIONS. The most important determinants of the gut microbiotic composition in infants were the mode of delivery, type of infant feeding, gestational age, infant hospitalization, and antibiotic use by the infant. Term infants who were born vaginally at home and were breastfed exclusively seemed to have the most "beneficial" gut microbiota (highest numbers of bifidobacteria and lowest numbers of *C difficile* and *E coli*).

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Key Words
infant, intestinal microbiota, bacterial colonization, 16S ribosomal RNA, real-time polymerase chain reaction

Abbreviations
PCR—polymerase chain reaction
CFU—colony-forming units
OR—odds ratio
CI—confidence interval

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THE GUT MICROBIOTA plays an important role in human health by providing a barrier for colonization of pathogens, by exerting important metabolic functions (fermentation of nondigestible fibers, salvage of energy as short-chain fatty acids, and production of vitamin K), and by stimulating the development of the immune system.[1] Bifidobacteria and lactobacilli are considered the most important health-beneficial bacteria for the human host, whereas bacteria such as staphylococci and clostridia are potentially pathogenic.[2] Because the gut microbiota is involved in many aspects of human health, it is important to understand how the composition of this microbial ecology is established.

During early life, there are major changes in the composition of the intestinal microbiota. At birth, the intestines are sterile. Within a few hours, bacteria start to appear in the feces. Because the intestinal environment of neonates shows a positive oxidation/reduction potential at birth, the gastrointestinal tract is colonized first by facultative aerobes. Gradually, the consumption of oxygen by these bacteria changes the intestinal environment into a more-reduced one, permitting the subsequent growth of strict anaerobes.[3]

The bacteria colonizing the infant gut during the first days of life originate mainly from the mother and the environment. In early life, one of the first major determinants of the gut microbiota is the mode of delivery. Vaginally born infants are colonized at first by fecal and vaginal bacteria of the mother, whereas infants born through cesarean section are exposed initially to bacteria originating from the hospital environment and health care workers.[3,4] Other factors that can influence the composition of the intestinal microbiota in neonates are the environment during birth, prematurity, hygiene measures, and the type of infant feeding.[5] We showed previously that breastfed infants had less *Clostridium difficile* and *Escherichia coli* in their feces at the age of 1 month, compared with formula-fed infants, whereas counts of bifidobacteria were comparable in the 2 groups.[6]

Although determinants of gut microbiotic composition were investigated previously in several studies, those studies focused on only 1 or a few determinants at a time and generally involved a limited number of infants.[7–11] Many potential determinants of the intestinal microbiota are clustered. For example, infants born through cesarean section need to stay in the hospital more often and receive antibiotics more frequently than do infants born vaginally. Consequently, the effects of individual determinants on the composition of the intestinal microbiota can be distinguished only if a large population is studied.

The aim of this study was to examine the influence of a broad range of potential determinants of gut microbiotic composition in a prospective cohort study in the Netherlands. Fecal samples of >1000 infants, 1 month of age, were analyzed with real-time polymerase chain reaction (PCR) assays, to detect quantitatively several bacterial groups and species.

METHODS

Subjects and Study Design

The KOALA Birth Cohort Study is a prospective birth cohort in the Netherlands. The design of this study was described in detail elsewhere.[12] Briefly, from October 2000 until December 2002, we recruited participants with diverse lifestyles, at 34 weeks of gestation. Pregnant women with a conventional lifestyle ($N = 2343$) were recruited from an ongoing prospective cohort study on pregnancy-related pelvic girdle pain in the Netherlands.[13] Pregnant women with an alternative lifestyle ($N = 491$) were recruited through organic food shops, anthroposophic doctors and midwives, Steiner schools, and magazines.

Beginning halfway during recruitment of the cohort (subjects recruited from January 2002 onward), fecal samples were collected from infants ($N = 1176$) at the age of 1 month. Subjects received a feces tube with spoon (Sarstedt, Nümbrecht, Germany), together with a sanitary napkin, an instruction form, and a brief questionnaire (feces questionnaire). Parents collected a fecal sample by placing a sanitary napkin in the diaper (to prevent absorption of the feces by the diaper) and collecting the feces out of the napkin into the collection tube, and they sent the sample as soon as possible (the same day) to the Department of Medical Microbiology at the University Hospital of Maastricht, by mail. Exclusion criteria were insufficient amount of feces (<1 g), feces collected before the age of 3 weeks or after the age of 6 weeks, and missing feces questionnaire.

Information on Potential Determinants

During pregnancy and the first months of the infant's life, information on perinatal determinants of the child's health, as well as hygiene, infections, nutrition, child rearing, and other lifestyle characteristics, was collected for all members of the cohort with repeated questionnaires. On the basis of these questionnaires, the following variables were selected as potential determinants of gut microbiotic composition: maternal education (lower education, vocational education, higher general secondary/preuniversity education, or higher vocational/academic education); maternal diet, defined as (1) regular diet (>50% of meat, eggs, vegetables, fruit, and milk of regular origin), (2) vegetarian diet (no meat but other products mainly of regular origin), (3) organic/biodynamic diet (>50% of meat, eggs, vegetables, fruit, and milk of organic or biodynamic origin), or (4) organic/biodynamic vegetarian diet; maternal probiotic use during pregnancy (frequency of consumption of dairy products with additional lactic acid-producing bacteria);

maternal antibiotic use during last month of pregnancy (yes or no); prolonged rupture of membranes (<24 or >24 hours before delivery); place and mode of delivery (vaginal delivery at home, vaginal delivery in hospital, artificial delivery [forceps delivery and vacuum extraction] in hospital, or cesarean section in hospital); hospitalization of the infant after birth (days of hospitalization immediately after birth); infant gender (male or female); gestational age (<37, 37–41, or >41 weeks); birth weight (<2500, 2500–4500, or >4500 g); birth season (winter, spring, summer, or autumn); type of infant feeding during the first 1 month of life (exclusively breastfed, exclusively formula fed, or a combination); brand of infant formula (exclusive consumption of brand A, brand B with locust bean gum, brand C, or brand D with oligosaccharides during the first 1 month of life); antibiotic/antifungal use by the infant during the first 1 month of life (yes or no); fever of the infant in the first 1 month of life (yes or no); number of siblings (0, 1 or 2, or >2); living on a farm (yes or no); and having furry pets (none, cat, dog, other, or a combination).

DNA Purification From Feces

At the laboratory, fecal samples were 10-fold diluted in peptone/water (Oxoid CM0009) containing 20% (vol/vol) glycerol (Merck, Darmstadt, Germany) and were stored at −20°C until analysis. For DNA isolation, 0.2 mL of the diluted feces was added to a 2-mL vial containing ~300 mg of glass beads (diameter: 0.1 mm) and 1.4 mL of ASL buffer from the QIAamp DNA stool mini-kit (Qiagen, Hilden, Germany), and the samples were disrupted in a mini-bead beater (Biospec Products, Bartlesville, OK) 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.

Microbial Analysis With Real-Time PCR Assays

DNA from all fecal samples was subjected to real-time PCR assays for bifidobacteria, *E coli*, *C difficile*, *Bacteroides fragilis* group, lactobacilli, and total bacteria based on 16S rDNA gene sequences (primers and probes are listed in Table 1). Development and validation of the real-time PCR assays were described in detail elsewhere.[6,14–17] The 5′-nuclease technique was used for detection of bifidobacteria, *E coli*, *C difficile*, and members of the *B fragilis* group. For detection of bifidobacteria, amplifications were conducted in a total volume of 50 μL, containing 1× TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA), 300 nmol/L levels of both primers, 150 nmol/L TaqMan probe, and 20 μL of purified target DNA (see above). For *E coli*, *C difficile*, and *B fragilis* group, amplifications were conducted in a total volume of 25 μL, containing 1× TaqMan Universal PCR Master Mix (Applied Biosystems), 900 nmol/L levels of both primers, 200 nmol/L TaqMan probe, and 10 μL of purified target DNA. The amplification (2 minutes at 50°C, 10 minutes at 95°C, and 42 cycles of 15 seconds at 95°C and 1 minute at 60°C) and detection were conducted with an Applied Biosystems Prism 7000 sequence detection system (Applied Biosystems).

For quantification of lactobacilli and total bacterial load, real-time detection of PCR products was conducted with SYBR Green I (Bio-Rad Laboratories, Hercules, CA). The PCR for lactobacilli was conducted in a total volume of 25 μL, containing 1× iQ SYBR Green Supermix (Bio-Rad), 500 nmol/L levels of both primers, and 5 μL of purified target DNA. The amplification was conducted as follows: 5 minutes at 95°C, followed by 35 cycles consisting of 15 seconds at 95°C, 20 seconds at 58°C, and 45 seconds at 72°C, with a final extension step at 72°C for 5 minutes. After amplification, melting curve analysis was performed from 60°C to 95°C, with increments of 0.5°C per 10 seconds. For quantification of the

TABLE 1 **Primers and Probes Used in This Study**

Target Organisms (Amplicon Size)	Primer/Probe	Sequence (5′ to 3′)	T_m, °C	Source
Bifidobacterium spp (126 bp)	Forward primer	GCGTGCTTAACACATGCAAGTC	59	Ref 6
	Reverse primer	CACCCGTTTCCAGGAGCTATT	59	Ref 6
	Probe	TCACGCATTACTCACCCGTTCGCC	70	Ref 6
E coli (96 bp)	Forward primer	CATGCCGCGTGTATGAAGAA	59	Ref 14
	Reverse primer	CGGGTAACGTCAATGAGCAAA	59	Ref 14
	Probe	TATTAACTTTACTCCCTTCCTCCCCGCTGAA	68	Ref 14
C difficile (114 bp)	Forward primer	TTGAGCGATTTACTTCGGTAAAGA	58	Ref 6
	Reverse primer	TGTACTGGCTCACCTTTGATATTCA	59	Ref 6
	Probe	CCACGCGTTACTCACCCGTCCG	69	Ref 6
B fragilis group (92 bp)	Forward primer	CGGAGGATCCGAGCGTTA	58	This study
	Reverse primer	CCGCAAACTTTCACAACTGACTTA	59	Ref 43
	Probe	CGCTCCCTTTAAACCCAATAAATCCGG	68	This study
Lactobacillus spp (341 bp)	Forward primer	AGCAGTAGGGAATCTTCCA	59	Refs 16 and 44
	Reverse primer	CACCGCTACACATGGAG	59	Refs 16 and 45
Total count (467 bp)	Forward primer	TCCTACGGGAGGCAGCAGT	59	Ref 17
	Reverse primer	GGACTACCAGGGTATCTAATCCTGTT	58	Ref 17

T_m indicates melting temperature; bp, base pairs.

total bacterial load, amplifications were conducted in a total volume of 25 μL, containing 1× iQ SYBR Green Supermix (Bio-Rad), 300 nmol/L levels of both primers, and 5 μL of purified target DNA. The amplification consisted of 4 minutes at 95°C and 30 seconds at 60°C, followed by 35 cycles of 30 seconds at 60°C, 15 seconds at 95°C, and 30 seconds at 60°C. Finally, melting curve analysis was performed from 60°C to 95°C, with increments of 0.5°C per 10 seconds. Amplification, melting curve analysis, and detection were conducted with the MyiQ single-color, real-time PCR detection system (Bio-Rad).

Statistical Analyses

Values of $\log_{10}$ colony-forming units (CFU) per gram for the bacterial groups and species were calculated for each stool sample from the threshold cycle values by using the constructed standard curves. The prevalence of colonization was expressed as the percentage of infants colonized with a specific bacterial group or species. To determine the unadjusted overall effects of the individual determinants on the prevalence and counts of the bacteria simultaneously, the Mann-Whitney rank-sum test was used.

To analyze the effects of the individual determinants, with adjustment for all other determinants, 2 multivariate approaches were used. First, linear regression analyses were used to examine the effects of the determinants on the bacterial counts. In the linear regression analyses, only infants who were colonized with the bacterial group or species were included. Total bacterial count was added as an additional independent variable to account for differences in the consistency of fecal samples. Second, logistic regression analyses were used to examine the effect of the determinants on the prevalence of colonization (colonized compared with not colonized). Both linear and logistic regression models included all of the determinants under study as independent variables and 1 of the bacterial groups or species as the dependent variable at a time. Length of hospitalization (in days) was included as a continuous variable in the regression models, whereas all other independent variables were incorporated as categorical variables.

To limit the chance of falsely rejecting the null hypothesis (no association) as a result of multiple testing, we chose to set α at .01 (2-sided), instead of the usual .05. Therefore, we present 99% confidence intervals (CIs) for the odds ratios (ORs) from logistic regression analyses.

Ethical Considerations

The KOALA Study was approved by the ethics committee of the University Hospital of Maastricht, and all parents signed informed consent for the study.

RESULTS

Study Samples

Fecal samples from a total of 1176 infants were collected. After exclusion of samples that were of insufficient amount ($n = 65$), samples that were collected before the age of 3 weeks or after the age of 6 weeks ($n = 54$), and samples for which the feces questionnaire was missing ($n = 25$), fecal samples from 1032 infants were included for analysis.

Almost all infants were colonized with bifidobacteria, and these bacteria outnumbered all other bacterial groups and species (Table 2). The majority of infants were also colonized with *E coli* and members of the *B fragilis* group, whereas both the prevalence and counts of lactobacilli and *C difficile* were much lower.

Table 3 shows the prevalence and counts of the bacterial groups and species for the individual determinants under study. In Table 4, the associations between these determinants and the prevalence and counts of the fecal bacteria under study, as determined in the multivariate analyses, are presented. More-detailed information on these associations, including regression coefficients and ORs from the linear and logistic regression analyses, is shown in Table 5.

Maternal Education, Diet, Antibiotic Use, and Probiotic Use

More than one half of the women in this population ($n = 572$) had a higher vocational or academic degree. Maternal education was not associated, however, with the infants' gut microbiotic composition.

Infants whose mothers consumed an organic or biodynamic diet seemed to have slightly lower numbers of *E coli* in their stools than did infants whose mothers consumed a regular diet (Table 3); however, this association was not observed in the multivariate analysis (data not shown). Mothers who consumed an organic diet more often (95%) breastfed their infants exclusively, compared with mothers who consumed a regular diet (59%), which suggests that the type of infant feeding was the underlying cause of the association between maternal diet and infant's microbiotic composition in the univariate analysis. Probiotic use and antibiotic use by

TABLE 2 **Median Counts and Prevalence of Selected Gut Bacteria in Feces of Infants 1 Month of Age ($n = 1032$)**

	Bifidobacteria	*E coli*	*C difficile*	*B fragilis* Group	Lactobacilli	Total
Median counts (range), $\log_{10}$ CFU/g feces	10.68 (6.84–11.56)	9.35 (5.91–10.79)	5.32 (2.70–9.57)	9.28 (5.74–10.44)	8.66 (7.92–10.73)	11.12 (9.43–12.14)
Prevalence, %	98.6	87.7	25.0	81.6	32.4	100

TABLE 3 **Median Counts and Prevalence of Colonization With Selected Gut Bacteria in Feces of Infants 1 Month of Age ($n = 1032$)**

Characteristics	No.	Bifidobacteria		*E coli*		*C difficile*		*B fragilis* Group		Lactobacilli		Total Counts, Median, $\log_{10}$ CFU/g Feces
		Counts, Median, $\log_{10}$ CFU/g Feces	Prevalence, %	Counts, Median, $\log_{10}$ CFU/g Feces	Prevalence, %	Counts, Median, $\log_{10}$ CFU/g Feces	Prevalence, %	Counts, Median, $\log_{10}$ CFU/g Feces	Prevalence, %	Counts, Median, $\log_{10}$ CFU/g Feces	Prevalence, %	
Maternal education												
Lower education[a]	75	10.71	100	9.47	92	4.95	24	9.32	77	8.87	39	11.16
Vocational	242	10.73	98	9.43	91	6.37	25	9.41	86	8.65	34	11.18
Higher general secondary/preuniversity	107	10.68	97	9.41	88	5.49	19	9.29	80	8.66	31	11.06
Higher vocational/academic education	572	10.65	99	9.24	86	5.30	25	9.21	80	8.64	30	11.08
Maternal diet[b]												
Regular[a]	723	10.67	99	9.49	89	5.40	26	9.39	82	8.70	34	11.15
Organic/biodynamic	49	10.56	98	8.91	82[c]	6.70	16	8.93	75	8.51	33	10.92
Vegetarian	47	10.63	98	9.06	85	6.76	17	9.00	81	8.66	32	11.05
Organic/biodynamic vegetarian	16	10.61	94	8.66	88	4.17	25	8.16	69	8.48	38	10.91
Maternal probiotic use[b]												
Never/sporadic[a]	814	10.67	99	9.30	88	5.06	25	9.24	82	8.64	32	11.10
Several times per month	100	10.62	98	9.23	82	6.74	21	9.36	75	8.68	34	11.12
Several times per week	65	10.75	100	9.45	95	5.30	39	9.29	83	8.74	39	11.24
Daily	23	10.48	100	9.71	70	5.40	13	9.34	83	8.71	35	11.21
Maternal antibiotic use[d]												
No[a]	972	10.68	99	9.32	88	5.10	25	9.28	82	8.65	32	11.12
Yes	38	10.60	92	9.48	95	5.92	16	9.30	74	8.68	50	11.13
Rupture of membranes												
≤24 h[a]	996	10.67	99	9.36	88	5.30	25	9.28	82	8.68	32	11.12
>24 h	36	10.61	100	8.93	89	6.97	19	9.23	78	8.50	31	11.05
Place and mode of delivery												
Natural delivery at home[a]	480	10.67	99	9.09	85	4.20	19	9.21	83	8.58	32	11.02
Natural delivery in hospital	346	10.74	99	9.54	91[e]	5.45	26[c]	9.40	85	8.65	34	11.18[e]
Artificial delivery in hospital	76	10.80	100	9.73	91[e]	6.19	34[c]	9.60	87	8.50	30	11.36[e]
Cesarean section in hospital	108	10.38	96[e]	9.59	88[c]	6.36	42[e]	6.67	63[e]	8.87	32	11.11
Hospitalization after birth												
No[a]	737	10.69	99	9.24	87	4.38	20	9.27	83	8.58	32	11.10
1 d	91	10.77	100	9.57	90	6.60	30	9.60	81	8.68	33	11.33[c]
2–3 d	85	10.65	100	9.72	93[e]	6.39	40[e]	9.41	79	8.88	37	11.23
4–6 d	73	10.36	96[e]	9.76	89	6.03	43[e]	6.56	68[e]	8.71	41	11.08
≥7 d	25	10.25	96[c]	9.40	88	6.94	40[c]	8.42	80	9.44	28	10.96
Gender												
Male[a]	531	10.64	99	9.37	89	5.61	24	9.20	80	8.76	32	11.10
Female	501	10.70	98	9.28	86	5.06	25	9.34	83	8.58	33	11.12
Gestational age at birth												
<37 wk (premature)	11	10.53	91	9.02	73	7.12	64[c]	8.95	82	8.80	27	10.80
37–41 wk[a]	860	10.68	99	9.30	87	5.06	23	9.27	82	8.61	33	11.12
>41 wk (postmature)	37	10.44	100	9.77	84	6.99	35	9.51	78	8.72	32	11.14
Birth weight												
<2500 g	11	10.45	100	8.67	100	7.12	27	9.31	82	9.12	36	11.28
2500–4500 g[a]	906	10.67	99	9.32	87	5.08	24	9.30	82	8.65	33	11.12
>4500 g	26	10.61	100	9.59	85	6.07	23	9.30	77	8.60	35	11.03
Birth season												
Winter[a]	285	10.67	99	9.37	85	5.61	22	9.20	81	8.65	32	11.11
Spring	241	10.65	96	9.26	88	6.28	25	9.44	82	8.87	37	11.13
Summer	286	10.55	99	9.23	92	4.60	23	9.04	82	8.65	33	11.04
Autumn	216	10.84	99	9.53	86	4.74	30	9.31	81	8.40	26	11.21

TABLE 3 **Continued**

Characteristics	No.	Bifidobacteria		*E coli*		*C difficile*		*B fragilis* Group		Lactobacilli		Total Counts, Median, log_{10} CFU/g Feces
		Counts, Median, log_{10} CFU/g Feces	Prevalence, %	Counts, Median, log_{10} CFU/g Feces	Prevalence, %	Counts, Median, log_{10} CFU/g Feces	Prevalence, %	Counts, Median, log_{10} CFU/g Feces	Prevalence, %	Counts, Median, log_{10} CFU/g Feces	Prevalence, %	
Type of infant feeding												
Exclusively breastfed[a]	700	10.67	99	9.06	85	4.53	21	8.99	79	8.54	29	10.98
Exclusively formula fed	232	10.69	97	9.84	94[e]	7.43	33[e]	9.76	88[e]	8.93	41[e]	11.43[e]
Combination	98	10.78	99	9.76	93[e]	5.58	35[c]	9.53	83[c]	8.71	34	11.36[e]
Type of infant formula												
Brand A[a]	47	10.51	96	9.83	91	7.68	40	9.84	89	8.84	34	11.45
Brand B (with locust bean gum)	19	10.80	95	9.81	89	6.56	21	9.80	95	8.40	47	11.43
Brand C	39	10.81	95	9.82	93	7.28	25	9.70	87	8.68	38	11.28
Brand D (with oligosaccharides)	20	11.19	100[e]	9.63	95	6.23	30	10.11	75	9.29	55	11.65
Antibiotic/antimycotic use during first 1 mo												
No[a]	945	10.70	98	9.32	88	5.50	25	9.31	82	8.65	32	11.12
Oral antibiotic	28	10.29	100[c]	9.45	79	7.12	18	6.39	82[c]	8.62	36	10.92
Oral miconazole	22	10.18	100[c]	9.57	82	4.47	23	9.35	86	8.68	36	11.04
Oral nystatin	15	10.77	93	9.67	87	4.81	13	9.33	73	8.64	33	11.00
Fever in first 1 mo												
No[a]	893	10.67	99	9.31	87	5.07	26	9.25	81	8.61	31	11.11
Yes	47	10.58	100	9.37	87	6.89	11	8.95	81	8.90	38	11.08
Siblings												
None[a]	414	10.63	98	9.51	86	6.10	27	9.40	80	8.77	34	11.21
1	452	10.70	99	9.27	88	5.06	23	9.21	82	8.64	31	11.07[c]
≥2	165	10.69	100	9.23	90	3.85	25	8.95	82	8.57	33	11.03[c]
Living on a farm												
No[a]	979	10.67	99	9.32	87	5.54	25	9.27	81	8.69	32	11.12
Yes	23	10.87	100	9.52	96	4.04	35	9.51	83	8.70	26	11.35
Furry pets												
None[a]	552	10.70	98	9.30	89	5.40	27	9.23	83	8.63	32	11.12
Dog	217	10.74	98	9.28	87	4.71	22	9.27	82	8.72	31	11.08
Cat	164	10.62	98	9.57	83	6.57	23	9.39	75	8.73	34	11.15
Other	35	10.71	97	9.19	89	4.88	11	9.29	83	8.23	23	10.94
Combination	64	10.56	100	9.43	94	5.17	28	9.36	83	8.75	41	11.14

Totals may not add up to 1032 because of missing values. Counts were calculated from positive samples only.

[a] Reference category.

[b] Consumed during pregnancy.

[c] $P < .01$, as determined with the Mann-Whitney rank-sum test, calculated from all samples (the statistical significance refers to an overall difference incorporating both counts and prevalence).

[d] Used somewhere during the last month of pregnancy.

[e] $P < .001$, as determined with the Mann-Whitney rank-sum test, calculated from all samples (the statistical significance refers to an overall difference incorporating both counts and prevalence).

the mother during pregnancy had no influence on the infant's gut microbiotic composition.

Delivery and Birth Characteristics

Amniotic membranes ruptured >24 hours before delivery for only 36 women. We found no association between prolonged rupture of membranes among women and the gut microbiotic composition of their infants.

More than one half of the infants were born in the hospital, and ~10% of all infants were born through cesarean section. In comparison with vaginal delivery at home, cesarean section resulted in lower colonization rates and counts of bifidobacteria and *B fragilis*-group species, whereas prevalence and counts of *C difficile* and counts of *E coli* were higher. The most-pronounced differences in colonization were seen for the *B fragilis* group and *C difficile*; compared with infants born vaginally at home, the median counts of *B fragilis*-group bacteria and *C difficile* were ~100-fold lower and ~100-fold higher, respectively, for infants born through cesarean section (Table 3). After adjustment for the other determinants, cesarean section was still associated with lower counts of bifidobacteria, a lower colonization rate and counts of *B fragilis*-group species, and higher counts of *C difficile*. In contrast, counts and colonization rate of *E coli* were no longer associated with cesarean section in the adjusted analyses. This is illustrated in Table 5 by a regression coefficient for counts close to 0 and an OR for prevalence close to 1.0. Although vaginal delivery and artificial delivery in the hospital seemed to be associated with

TABLE 4 **Associations Between Determinants and Selected Gut Bacteria in Feces of Infants 1 Month of Age, as Determined in Multivariate Analyses**

	Association									
	Bifidobacteria		*E coli*		*C difficile*		*B fragilis* Group		Lactobacilli	
	Counts	Prevalence	Counts	Prevalence	Counts	Prevalence	Counts	Prevalence	Counts	Prevalence
Cesarean section (compared with vaginal delivery)	−−	ND				+	−−	−−		
Hospitalization (d)		ND				+				
Prematurity (compared with term infants)		ND			+					
Exclusive formula feeding (compared with exclusive breastfeeding)		ND		+	+	++		+		+
Antibiotic use by infant (yes/no)	−	ND					−−			
Miconazole use by infant (yes/no)	−	ND								
Siblings (yes/no)	+	ND								

Results for determinants with no statistically significant results for any of the bacteria are not presented. Associations between determinants and counts were examined with linear regression analyses; models included the following independent variables: maternal education, maternal diet, maternal probiotic use, maternal antibiotic use, rupture of membranes, place and mode of delivery, gender, gestational age, birth weight, birth season, hospitalization after birth, type of infant feeding, antibiotic/antimycotic use by infant, fever of infant, siblings, farm residence, furry pets, and total bacterial count. Associations between determinants and prevalence of colonization (colonized compared to not colonized) were examined with logistic regression analyses; models included the following variables: maternal education, maternal diet, maternal probiotic use, maternal antibiotic use, rupture of membranes, place and mode of delivery, gender, gestational age, birth weight, birth season, hospitalization after birth, type of infant feeding, antibiotic/antimycotic use by infant, fever of infant, siblings, farm residence, and furry pets. ND indicates not determined (logistic regression analysis of prevalence of bifidobacteria was not performed because 99% of infants were colonized); +, positive association, $P < .01$; ++, positive association, $P < .001$; −, negative association, $P < .01$; −−, negative association, $P < .001$.

higher counts and prevalences of *E coli* and *C difficile*, compared with home birth (Table 3), these associations were present only in the unadjusted analyses.

Hospitalization after birth was associated only with a higher *C difficile* colonization rate after controlling for other determinants. The prevalence of *C difficile* increased ~13% per day of hospitalization, compared with nonhospitalized infants (OR: 1.13; 99% CI: 1.01–1.25).

Only 11 infants in this study population were born premature (<37 weeks). These infants were colonized more often with *C difficile* (64%, as opposed to 23%), with considerably higher counts (7.12 $\log_{10}$ CFU/g feces, compared with 5.06 $\log_{10}$ CFU/g feces), compared with term infants (Table 3). With adjustment for other determinants, the counts were still statistically significantly higher (Tables 4 and 5). The OR was still consistent with a higher colonization rate (OR: 4.47) but was not statistically significant, probably because of the low power caused by the small number of infants. No association was found between the gut microbiota and birth weight, birth season, or gender of the infant.

Infant Feeding

Most infants ($n = 700$) were breastfed exclusively up to the first 1 month of life, whereas 232 infants were formula fed exclusively and 98 infants received a combination of breastfeeding and formula feeding. Exclusively formula-fed infants were more often colonized with *E coli*, *C difficile*, *B fragilis* group, and lactobacilli than were their exclusively breastfed counterparts, as shown in both the unadjusted (Table 3) and adjusted (Tables 4 and 5) analyses. The counts of *E coli*, *C difficile*, *B fragilis* group, and lactobacilli were also significantly higher for formula-fed infants, compared with breastfed infants, in the unadjusted analyses. In the adjusted analyses, only counts of *C difficile* were still significantly higher for formula-fed infants, whereas counts of both *E coli* ($P = .03$) and *B fragilis* ($P = .027$) still tended to be higher for formula-fed infants (not reaching the level of significance of $P < .01$) (Table 5).

In our population, 4 brands of formula were used frequently. Brand B contained locust bean gum and brand D was enriched with oligosaccharides, whereas the others were not. Infants fed exclusively with 1 of these 4 formulas were compared. As shown in Table 3, infants fed the oligosaccharide-enriched formula (brand D) harbored greater numbers of bifidobacteria in their stools. After adjustment for the other determinants under study, counts of bifidobacteria (coefficient: 0.60; $P = .04$) and also counts of lactobacilli (coefficient: 0.75; $P = .02$) tended to be higher for infants fed formula D, compared with reference formula A (data not shown).

Antibiotics, Antimycotic Agents, and Fever

Oral use of antibiotics (mainly amoxicillin) by the infant during the first 1 month of life resulted in decreased numbers of bifidobacteria and *B fragilis*-group species. Lower counts of bifidobacteria were also observed after oral administration of the antimycotic miconazole (Tables 3 and 4). Infants who experienced a fever in their first 1 month of life did not have different gut microbiotic composition, compared with infants without a fever.

TABLE 5 Linear Regression Coefficients for Bacterial Counts and ORs for Presence of Gut Bacteria, With Respect to Determinants in Multivariate Analyses

	Bifidobacteria		*E coli*		*C difficile*		*B fragilis* Group		Lactobacilli	
	Coefficient (*P*)	OR (99% CI)	Coefficient (*P*)	OR (99% CI)	Coefficient (*P*)	OR (99% CI)	Coefficient (*P*)	OR (99% CI)	Coefficient (*P*)	OR (99% CI)
Cesarean section (compared with vaginal delivery)	−0.34 (.003)[a]	ND	0.07 (.677)	1.04 (0.38–2.83)	0.88 (.24)	2.07 (1.01–4.25)[a]	−1.36 (<.001)[a]	0.28 (0.13–0.61)[a]	0.31 (.032)	0.84 (0.42–1.70)
Hospitalization (d)	−0.01 (.365)	ND	0.04 (.108)	1.00 (0.86–1.17)	0.06 (.364)	1.13 (1.01–1.25)[a]	0.01 (.621)	1.02 (0.90–1.16)	0.02 (.306)	1.02 (0.92–1.12)
Prematurity (compared with term infants)	0.38 (.282)	ND	−0.81 (.109)	0.11 (0.01–1.15)	2.83 (.007)[a]	4.47 (0.48–41.85)	0.38 (.432)	0.96 (0.09–10.38)	−0.23 (.580)	0.68 (0.09–5.25)
Exclusive formula-fed (compared with exclusively breastfed)	−0.10 (.233)	ND	0.24 (.031)	2.90 (1.22–6.89)[a]	1.03 (.003)[a]	1.88 (1.13–3.11)[a]	0.25 (.027)	2.22 (1.16–4.24)[a]	0.056 (.564)	1.64 (1.03–2.60)[a]
Antibiotic use by infant (yes/no)	−0.66 (.001)[a]	ND	0.06 (.825)	0.57 (0.12–2.66)	0.94 (.324)	0.59 (0.13–2.75)	−1.10 (<.001)[a]	1.30 (0.27–6.19)	−0.16 (.470)	1.11 (0.34–3.63)
Miconazole use by infant (yes/no)	−0.59 (.003)[a]	ND	0.41 (.142)	0.60 (0.13–2.90)	0.04 (.965)	1.01 (0.25–4.09)	0.174 (.506)	1.49 (0.27–8.20)	0.17 (.468)	1.27 (0.38–4.25)
Siblings (yes/no)	0.25 (.001)[a]	ND	0.21 (<.025)	1.45 (0.82–2.57)	−0.32 (.277)	1.01 (0.66–1.56)	0.004 (.907)	1.09 (0.68–1.74)	0.01 (.923)	0.88 (0.59–1.29)

Results for determinants with no statistically significant results for any of the bacteria are not presented. Coefficients are regression coefficients of association between determinants and counts, determined with linear regression analyses; models included the following independent variables: maternal education, maternal diet, maternal probiotic use, maternal antibiotic use, rupture of membranes, place and mode of delivery, infant gender, gestational age, birth weight, birth season, hospitalization after birth, type of infant feeding, antibiotic/antimycotic use by infant, fever of infant, siblings, farm residence, furry pets, and total bacterial counts. ORs of association between determinants and prevalence of colonization (colonized compared with not colonized) were determined with logistic regression analyses; models included the following variables: maternal education, maternal diet, maternal probiotic use, maternal antibiotic use, rupture of membranes, place and mode of delivery, infant gender, gestational age, birth weight, birth season, hospitalization after birth, type of infant feeding, antibiotic/antimycotic use by infant, fever of infant, siblings, farm residence, and furry pets. ND indicates not determined (logistic regression analysis of prevalence of bifidobacteria was not performed because 99% of infants were colonized).

[a] Statistically significant results (at $\alpha = .01$, 2-sided).

Home Environment

Infants with older siblings had lower total bacterial counts per gram of feces than did infants without siblings, as seen in the unadjusted analysis (Table 3). After adjustment for these differences in total bacterial counts and other potential differences between infants with and without siblings, a greater proportion of bifidobacteria was found for infants with older siblings, compared with infants without siblings (Tables 4 and 5). Infants' gut microbiota was not associated with farm residence or the presence of furry pets in the home, as determined in both the unadjusted (Table 3) and multivariate (data not shown) analyses.

DISCUSSION

To our knowledge, this is the first large, prospective, epidemiologic study on determinants of gut microbiotic composition in early infancy. As a consequence of the large number of infants in the KOALA study, we were able to study the potential determinants in a multivariate manner and to distinguish their independent effects. The fecal samples were analyzed with real-time quantitative PCR assays. This molecular approach can be applied to high-throughput analyses with frozen samples, which makes this technique very suitable for a large-scale epidemiologic study such as this. Furthermore, real-time quantitative PCR analyses overcome many of the limitations of traditional bacteriologic culture techniques, such as the low sensitivity, the low level of reproducibility because of the multitude of species to be identified and quantified, and the time-consuming aspects of the conventional methods.[14] However, molecular techniques based on amplification of 16S rDNA require that the microbial cells in the sample first be lysed for the extraction of DNA. There is a vast difference in the susceptibility of the cells of different microbial species to lytic procedures. When only 1 lytic method is used, it is unlikely that template DNA for the real-time PCR analysis is extracted with equal success from all species.[18] Therefore, we chose to add a mechanical lysis step to the chemical lyses of the Qiagen stool mini kit.

A drawback of this study was the time between collection of the samples by the parents and processing of the samples in the laboratory, which was ~1 day. Ott et al[19] demonstrated clearly that the total amount of bacterial DNA, as well as the diversity of the microbiota, decreased significantly over such a time period. However, they also showed that the similarity (determined with denaturing gradient gel electrophoresis) of fecal samples processed directly and those processed after 24 hours remained high. This means that the dominant microbiota seems to be relatively stable. Furthermore, the aim of the present population-based study was to examine differences in gut microbiotic composition between subjects.[19] It is not likely that the possible changes

in composition of the samples during transport were influenced by the determinants under study.

It was demonstrated previously that diet can have an influence on the gut microbiota. For example, introduction of an extreme vegan diet was shown to change bacterial fatty acid profiles in the feces.[20] Also, an organic diet (consumption of foods that are produced without the use of synthetic inputs, such as synthetic fertilizers and pesticides, veterinary drugs, genetically modified seeds and breeds, preservatives, additives, and irradiation) may influence the gut microbiota, because organically produced foods include spontaneously fermented vegetables containing lactobacilli.[21] We hypothesized that the maternal diet not only might be a determinant of the mother's gut microbiota but also might influence her infant's gut microbiotic composition. Indeed, maternal diet seemed to have some influence on the offspring's microbiota, as shown in the univariate analysis; *E coli* counts were lower and the prevalence and counts of *B fragilis*-group species tended to be lower for infants of mothers consuming organic diets. However, this association disappeared completely after adjustment for other determinants. This can be explained at least in part by the fact that mothers who consume an organic diet more often breastfeed their infants, compared with mothers with regular diets. This emphasizes clearly the need for multivariate analysis when the effects of determinants of gut microbiotic composition are studied.

We did not find an association between maternal use of probiotics during pregnancy and the intestinal microbiotic composition of the offspring at the age of 1 month. However, it is not unlikely that transfer of probiotic bacterial strains from mother to child occurs, especially when the child is exposed to maternal feces during vaginal delivery. Although probiotic bacteria do not colonize permanently the gut of adults,[22,23] this might be different for neonates, who do not yet have an established gut microbiota. Indeed, Schultz et al[24] confirmed such a transmission of probiotic strains from mother to child during birth; although the specific probiotic strain was present in very low numbers (10^4–10^5 CFU/g feces) for most infants, it persisted at least until the age of 6 months. In the present study, we did not investigate the presence of specific probiotic strains but focused on the total numbers of bifidobacteria and lactobacilli. Consequently, a putative small contribution of probiotic bacterial strains to the total number of lactobacilli or bifidobacteria would not be noticed. Furthermore, the pregnant women in our cohort used a wide range of different probiotic products, including different strains. This heterogeneity might explain why an association between probiotic use by the mothers and the gut microbiotic composition of the infants was not found. Firm conclusions regarding the effects of changes in the maternal microbiota itself cannot be made, however, because we have no data on the impact of maternal diet and probiotic use on the mother's own microbiota.

In addition to maternal diet and probiotic use, antibiotic use by the mother during the last month of pregnancy was selected as a potential determinant of the infant's microbiota. This was based on the notion that changes in maternal microbiota could affect the infant's microbiota. However, as for the previous 2 determinants, maternal antibiotic use was not found to be associated with any of the examined gut bacteria.

Prolonged rupture of amniotic membranes before delivery increases the risk of fetal infection, especially with group B streptococci.[25] However, we found no association between prolonged rupture of membranes and the commensal gut bacteria under study. Because the presence of streptococci was not examined, we cannot draw any conclusions about the association between prolonged rupture of membranes and the presence of this specific group of bacteria in the infant's gut.

The colonization rate and counts of the *B fragilis* group differed most markedly between vaginally delivered infants and infants born through cesarean section. This is in agreement with 2 previous studies, which also found levels of members of the *B fragilis* group to be greatly reduced as a result of cesarean section.[4,26] Furthermore, we found bifidobacterial counts to be lower (although this was less pronounced) and colonization rates of *C difficile* to be higher for infants born through cesarean section, compared with infants born vaginally at home.

After adjustment for potential confounding by the other determinants, the hospital environment itself had an effect only on the colonization rate of *C difficile*. Indeed, it is thought generally that infants are being colonized with this spore-forming, anaerobic, microorganism mainly through the hospital environment. It was reported previously that vaginal swabs collected just before delivery were uniformly negative for this organism.[27] In contrast, *C difficile* was isolated from hands and stools of healthy hospital personnel and from a NICU, where spores may persist for months.[28] Although carriage of *C difficile* by healthy adults is uncommon,[29] the relatively high colonization rates among asymptomatic infants found in our study are in accordance with findings from previous studies.[30–33] The highest carriage rate of *C difficile* in our birth cohort was among premature infants; 64% of the infants born before 37 weeks of gestation were colonized. Prematurity is associated strongly with hospitalization, which could explain in part the relatively high prevalence of *C difficile* in preterm infants. However, after controlling for other determinants, the counts of *C difficile* were still significantly higher in preterm infants than in term infants. Several factors that were not included in the present study may account for the higher numbers of *C difficile* in preterm infants, such as the immature gastrointestinal tract and delayed oral feeding.

Like previous studies,[11,34,35] we found that breastfed infants had a microbiota dominated by bifidobacteria, with rates of colonization with *E coli*, *C difficile*, *B fragilis*-group species, and lactobacilli that were significantly lower than those for formula-fed infants. Infants fed exclusively with a formula supplemented with a mixture of galactooligosaccharides and fructooligosaccharides (brand D) had higher counts of bifidobacteria and lactobacilli in their stools, compared with infants fed an unsupplemented formula. Although the compositions of the formulas differed not only in the oligosaccharide supplementation, it is well known that supplementation with oligosaccharides increases counts of lactic acid-producing bacteria.[36,37] As opposed to the time of this study, presently most formulas are supplemented with oligosaccharides in the Netherlands; therefore, differences in gut microbiotic composition as a result of different formulas probably have disappeared.

Although the use of antibiotics may have a major effect on the composition of the gut microbiota, the effect differs between antibiotics.[38] Because of the small number of infants in our cohort who received oral antibiotic therapy in their first 1 month, we were not able to distinguish between different antibiotics. Nevertheless, oral antibiotic administration had clear-cut effects on the anaerobic microbiota, with counts of bifidobacteria and *Bacteroides* being decreased.

In the unadjusted analyses, total bacterial counts were lower in infants with siblings than in first children. After adjustment for these differences in total bacterial counts and all other determinants, the proportion of bifidobacteria was significantly higher in infants with older siblings, compared with first children, and counts of *E coli* also tended to be higher in infants with older siblings. Again, this emphasizes the importance of multivariate analyses in studying a range of determinants of the gut microbiota. Studies on the etiology of allergic diseases have often found a protective effect of older siblings.[39] This sibling effect has been hypothesized to be a marker of early-life infections. However, direct evidence for this hypothesis is lacking.[40] Because allergic diseases have also been linked to aberrant gut microbiotic composition,[41,42] it has been suggested that this sibling effect in allergy could be partly attributable to the commensal gut microbiotic composition. To our knowledge, we are the first to demonstrate that having older siblings is indeed associated with the composition of the gut microbiota. It is clear, however, that the difference between children with and without siblings is small, and the clinical relevance of this difference remains to be elucidated.

CONCLUSIONS

Infant feeding had a major effect on the gut microbiotic composition in infants at the age of 1 month. Breastfed infants were less often colonized with bacteria other than bifidobacteria, compared with formula-fed infants. The effect of mode of delivery was also of major importance, especially regarding *Bacteroides*. Although passing through the birth canal (vaginal delivery) and thus coming in contact with maternal feces was associated strongly with the infant's gut microbiota, we found no effect on the infant's gut microbiota of determinants that potentially influence the microbial composition of maternal feces (maternal diet, antibiotic use, and probiotic use). Hospitalization and prematurity were both associated positively with *C difficile*. Antibiotic use reduced greatly the levels of obligate anaerobes (bifidobacteria and *Bacteroides*), whereas having older siblings was associated with slightly higher bifidobacterial counts. Term infants who were born vaginally at home and were breastfed exclusively seemed to have the most "beneficial" gut microbiota, with the highest numbers of bifidobacteria and lowest numbers of *C difficile* and *E coli*.

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Factors Influencing the Composition of the Intestinal Microbiota in Early Infancy

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