

Influence of Environmental and Genetic Factors Linked to Celiac Disease Risk on Infant Gut Colonization by *Bacteroides* Species[∇]

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Celiac disease (CD) is an immune-mediated enteropathy involving genetic and environmental factors whose interaction might influence disease risk. The aim of this study was to determine the effects of milk-feeding practices and the HLA-DQ genotype on intestinal colonization of *Bacteroides* species in infants at risk of CD development. This study included 75 full-term newborns with at least one first-degree relative suffering from CD. Infants were classified according to milk-feeding practice (breast-feeding or formula feeding) and HLA-DQ genotype (high or low genetic risk). Stools were analyzed at 7 days, 1 month, and 4 months by PCR and denaturing gradient gel electrophoresis (DGGE). The *Bacteroides* species diversity index was higher in formula-fed infants than in breast-fed infants. Breast-fed infants showed a higher prevalence of *Bacteroides uniformis* at 1 and 4 months of age, while formula-fed infants had a higher prevalence of *B. intestinalis* at all sampling times, of *B. caccae* at 7 days and 4 months, and of *B. plebeius* at 4 months. Infants with high genetic risk showed a higher prevalence of *B. vulgatus*, while those with low genetic risk showed a higher prevalence of *B. ovatus*, *B. plebeius*, and *B. uniformis*. Among breast-fed infants, the prevalence of *B. uniformis* was higher in those with low genetic risk than in those with high genetic risk. Among formula-fed infants, the prevalence of *B. ovatus* and *B. plebeius* was increased in those with low genetic risk, while the prevalence of *B. vulgatus* was higher in those with high genetic risk. The results indicate that both the type of milk feeding and the HLA-DQ genotype influence the colonization process of *Bacteroides* species, and possibly the disease risk.

The newborn intestine is colonized immediately after birth by microorganisms from the mother and the environment (12, 21). At birth, the intestinal milieu of neonates shows a positive redox potential, and early bacterial colonization begins with facultative anaerobes (*Enterobacteriaceae*, *Lactobacillus*, etc.) that gradually consume the oxygen, permitting the growth of strict anaerobes (*Bifidobacterium*, *Bacteroides*, *Clostridium*, etc.) (18, 22). Subsequently, milk-feeding practices play an important role in the microbiota composition of the infant gut (2, 18, 22). In breast-fed infants, the microbiota is less diverse and is dominated by *Bifidobacterium* species, while a more diverse microbiota develops only after complementary feeding commences. In contrast, the bacterial composition of formula-

fed infants is dominated by members of diverse genera (*Enterobacteriaceae*, *Streptococcus*, *Bacteroides*, *Clostridium*, and *Bifidobacterium*) (1, 9). Intestinal colonization influences diverse physiological functions, which may have an impact on the host's health and disease risk (16, 18, 25). Nevertheless, there is still limited information on the initial establishment of *Bacteroides* species and its possible influence on health (11, 19, 30).

Celiac disease (CD) is a multifactorial disorder involving both genetic and environmental factors. This disease is associated with human leukocyte antigen (HLA) genes of the major histocompatibility complex (MHC), and approximately 95% of patients are positive for HLA-DQ2 or -DQ8 (28). Studies of twins also showed that in 25% of cases, one twin of the pair did not develop CD, supporting the role of environmental factors in disease development (10, 20). Breast-feeding seems to exert a protective effect against CD development (4, 15), but its possible connection with modulation of the intestinal microbiota is unknown. A preliminary study suggested an association between increased *Bacteroides-Prevotella* group

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TABLE 1. Demographic characteristics of the study cohort of infants^a

Parameter	Value for low-risk infants (<1% probability of CD) (n = 36)		Value for high-risk infants (7–28% probability of CD) (n = 39)		P value
	Breast-fed (n = 20)	Formula-fed (n = 16)	Breast-fed (n = 20)	Formula-fed (n = 19)	
No. of infants with first-degree relative(s) with CD					0.923
Parent	4	2	5	3	
Sibling	14	12	12	14	
Parent and sibling	2	2	3	4	
No. of infants with delivery type					0.900
Vaginal	17	13	18	16	
Caesarean	3	3	2	3	
Length (cm)	49.2 ± 1.4	50.0 ± 1.9	49.6 ± 2.2	50.5 ± 1.7	0.309
Weight (g)	3,253.0 ± 307.7	3,269.0 ± 519.3	3,364.0 ± 514.7	3,459.0 ± 795.3	0.640
Length of gestation (wk)	38.3 ± 3.7	38.3 ± 1.3	39.3 ± 1.1	39.5 ± 1.4	0.332

^a Infants were classified into two main CD risk groups according to their HLA-DQ haplotype (3) and considering the HLA distribution of the Eastern Spanish population (5).

proportions and the HLA-DQ genotype, but the sample size was limited (8).

The aim of this study was to determine the influence of milk-feeding practices (breast-feeding versus formula feeding) and HLA-DQ genotype on the intestinal colonization process of *Bacteroides* species in a representative group of infants with a familial risk of developing CD. To do so, PCR and denaturing gradient gel electrophoresis (DGGE) analyses were performed. The final purpose of the study was to shed light on the interactions between the intestinal colonization process, diet, and genotype and on their overall influence on CD risk.

MATERIALS AND METHODS

Subjects. This study included 75 full-term newborns with at least one first-degree relative suffering from CD, selected from an ongoing prospective observational 3-year study. Seventy-four percent of the infants had a sibling suffering from CD, 17% of the infants had a parent with CD, and 9% of the infants had both one sibling and one parent with CD. The distributions of the type and number of relatives suffering from CD were similar for the subgroups of infants considered for statistical comparisons (Table 1). Fifty-one percent of the samples were from infants born in Madrid (center of Spain), and the rest (49%) were from infants born in Eastern Spain. Exclusion criteria included prematurity, maternal infections or clinical illness during pregnancy, maternal antibiotic or probiotic administration during the last 2 weeks of gestation and intrapartum, and babies given antibiotic prophylaxis or therapy. Infants were divided according to feeding practice, into formula-fed infants (n = 35), eligible if they were fed exclusively with formula from birth, and breast-fed infants (n = 40), eligible if they were breast-fed exclusively during the 4-month period under study. Infants were also classified into two main CD genetic risk groups, a high-risk group (n = 39) and a low-risk group (n = 36), on the basis of their HLA-DQ genotype. The classification was based on the criteria of Bourgey et al. (3) and considering the HLA distribution of the Eastern Spanish population (5). The demographic data and dietary history of every subject were recorded (Table 1). The study was conducted in accordance with the ethical rules of the Helsinki Declaration (Hong Kong revision, September 1989), following EEC Good Clinical Practice guidelines (document 111/3976/88, July 1990) and current Spanish law, which regulates clinical research in humans (Royal Decree 561/1993 regarding clinical trials). The study was approved by the local ethics committees of the CSIC and the hospitals involved (Hospital Universitario Sant Joan de Reus, Hospital Universitario Sant Joan de Deu, Hospital Clínico Universitario de Valladolid, Hospital Universitario La Paz, Hospital Infantil Universitario La Fe, Hospital Universitario Infantil Niño Jesús, and Hospital Universitario Nuestra Señora de

Candelaria). Written informed consent was obtained from the parents of children included in the study.

Bacterial strains. The reference strains used as ladders for *Bacteroides* species identification by DGGE were *Bacteroides distasonis* DSM 20701, *B. fragilis* DSM 2451, *B. ovatus* DSM 1896, *B. thetaiotaomicron* DSM 2079, and *B. vulgatus* DSM 1447. Another six strains used as ladders were isolated from human stools and identified by 16S rRNA gene sequencing with primers 27d and 1401r as described elsewhere (13), using an ABI Prism 3130XL genetic analyzer (Applied Biosystems, CA). Search analyses were conducted in GenBank, using the Basic Local Alignment Search Tool (BLAST) algorithm. Sequences of our isolates showed >97% similarity to sequences of the species *B. dorei*, *B. massiliensis*, *B. caccae*, *B. coprocola*, *B. intestinalis*, and *B. uniformis* (GenBank accession numbers EU722737.1, AB510703.1, AB510697.1, AB200223.1, AB437413.1, and AB247141.1, respectively) and were assigned to these species. *Bacteroides* strains were grown in Schaedler agar medium (Scharlau, Barcelona, Spain) supplemented with kanamycin (100 mg/liter), vancomycin (7.5 mg/liter), and vitamin K (0.5 mg/liter) and incubated under anaerobic conditions (AnaeroGen; Oxoid, Hampshire, United Kingdom) at 37°C.

DNA extraction, PCR amplification, and DGGE analysis. Stool samples were collected from every infant at 7 days, 1 month, and 4 months of age and kept at -20°C until analysis. Samples were diluted and homogenized. DNAs from diluted stool samples and from bacterial strains used as ladders were extracted by using a QIAamp DNA stool minikit (Qiagen, Hilden, Germany) following the manufacturer's instructions.

Bacteroides genus-specific PCR was performed with 16S rRNA gene-targeted primers Bfra531-f (5'-ATACGGAGGATCCGAGCGTTA-3') and Bfra766-GC-r (5'-CTGTTTGTATCCACACTGCCCGCCGCGCGGGCGGGCGGGGGGGGGGGG-3'). Each reaction mixture of 30 µl was composed of 3 µl 10× buffer stock (containing 1.5 mM MgCl₂), 1.5 µl bovine serum albumin (10 mg/ml), a 0.5 µM concentration of each deoxynucleoside triphosphate, 1 µM (each) primers, 2.5 U *Taq* polymerase (Ecotaq; Ecogen, Barcelona, Spain), and 30 ng DNA. DGGE analysis was carried out on a Dcode universal mutation detection system (Bio-Rad, Richmond, CA) (23). Unknown DGGE bands were excised from the gels, reamplified, and purity checked by DGGE. The PCR products were purified using a GFX PCR DNA and gel band DNA purification kit (GE Healthcare, Buckinghamshire, United Kingdom) and were identified by DNA sequencing using an ABI Prism 3130XL genetic analyzer (Applied Biosystems, CA). Search analyses were conducted as described above, and sequences with >97% similarity were considered to be of the same species.

Statistical and cluster analyses. Gels were aligned using ladders, and bands were estimated visually and coded as present or absent. The relatedness of microbial communities was expressed as similarity clusters, using the Dice coefficient and the unweighted-pair group method using average linkages (UPGMA), and the diversity of taxa present in fecal samples was calculated with the Shannon-Wiener index (*H'*), using PAST (Paleontological Statistics) soft-

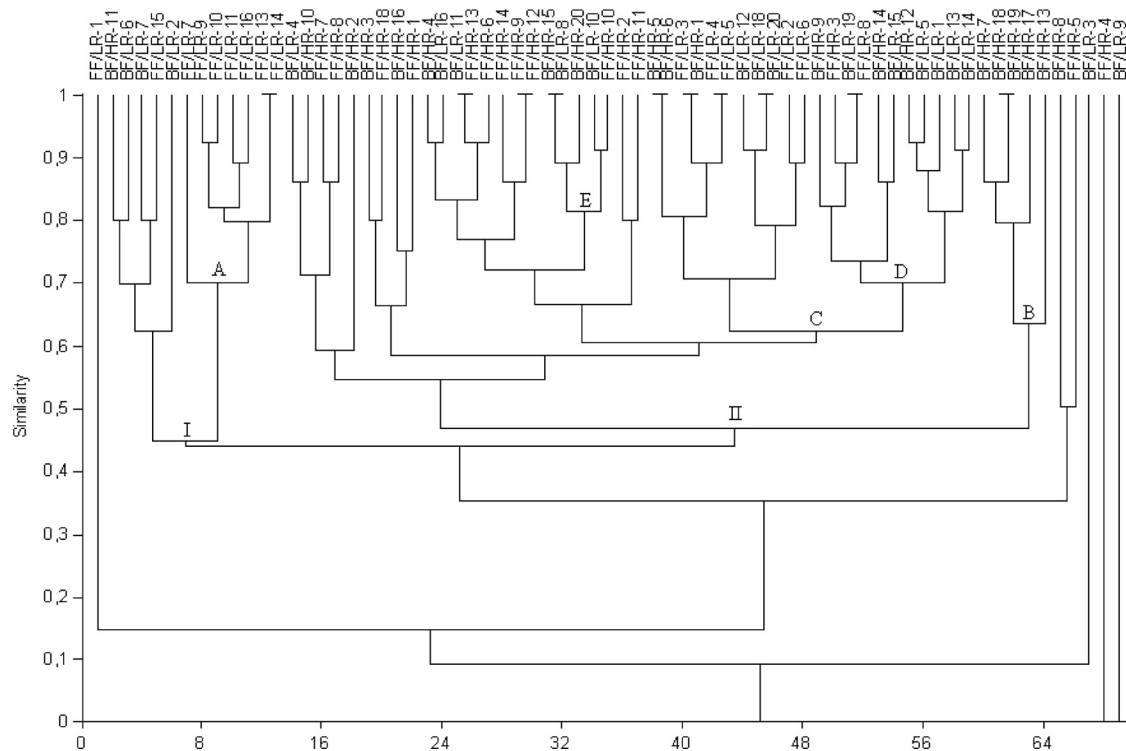


FIG. 1. Dendrogram derived from DGGE analysis of *Bacteroides* species in fecal samples of the study cohort of infants at 7 days of age, based on Dice's similarity index and the UPGMA clustering algorithm. Infants were divided according to feeding practice (breast-fed [BF] and formula-fed [FF]) and genetic risk of CD development (low risk [LR] and high risk [HR]). Letters correspond to clusters of samples.

ware. The Levene test was used to establish variance homogeneity and diversity data distributions, and the effects of feeding practices and genetic risk on *Bacteroides* diversity were determined by using a linear mixed-model analysis for repeated measures in which sampling time was the repeated variable, using SPSS 17.0 software (SPSS Inc., Chicago, IL). *P* values of <0.05 were considered statistically significant. The interaction between feeding practices and genetic risk of CD in the studied cohort of infants was not statistically significant by applying the linear mixed-model analysis. Differences in *Bacteroides* species prevalence were established by applying χ^2 tests and, where appropriate, by two-tailed Fisher's exact test. Analyses were carried out with Statgraphics software (Manugistics, Rockville, MD), and statistical differences were established in cases with *P* values of <0.05 .

RESULTS

Infant gut colonization by *Bacteroides* species during the first months of life. In considering the whole cohort of infants, significant differences in *Bacteroides* species diversity indexes (H') were not detected based on the infants' ages, with values of 1.45 at 7 days, 1.42 at 1 month, and 1.36 at 4 months.

Some profiles clustered according to infant age, but others were subject specific, so cluster analysis did not classify *Bacteroides* profiles according to infant age (data not shown), nor did the prevalence of *Bacteroides* species differ significantly in the cohort of infants at different ages (data not shown).

Influence of milk-feeding method on *Bacteroides* species colonization. The *Bacteroides* diversity index (H') was significantly higher for formula-fed infants than for breast-fed infants during the study period, as determined by applying a linear mixed-model analysis for repeated measures ($P = 0.01$).

Clustering analysis of DGGE profiles obtained for infant stools at 7 days, 1 month, and 4 months of age revealed some

associations between the types of milk-feeding practices. At 7 days of age, some breast-fed and formula-fed infant profiles were grouped together. Cluster A contained 7 formula-fed infant profiles (70% similarity). Moreover, another three clusters containing breast-fed infant profiles were found: cluster B contained 5 profiles, cluster D contained 9 profiles, and cluster E contained 4 profiles, with 63%, 70%, and 82% similarity, respectively (Fig. 1). At 1 month of age, cluster I included most of the breast-fed infant profiles (33), with 55% similarity, whereas cluster II contained 7 formula-fed infant profiles (65% similarity) (Fig. 2). At 4 months of age, 24 breast-fed infant profiles were grouped in cluster D (63% similarity). However, this cluster also contained another 2 subclusters (E and F) including 4 formula-fed infant profiles each, with similarities of $>80\%$. This analysis also revealed another cluster (B), grouping 8 formula-fed infant profiles at 54% similarity (Fig. 3).

The prevalences of *Bacteroides* species in the cohort of infants, given according to feeding practices, are shown in Table 2. At 7 days of age, the prevalence of *B. vulgatus* was higher in breast-fed infants than in formula-fed infants ($P = 0.03$). In contrast, *B. caccae* and *B. intestinalis* were detected more frequently in formula-fed infants than in breast-fed infants ($P < 0.01$). At 1 month of age, the prevalence rates of *B. vulgatus* and *B. caccae* were equal in both the breast-fed and formula-fed groups; nevertheless, the prevalence of *B. intestinalis* continued to be increased in formula-fed infants compared with breast-fed infants ($P < 0.01$). In contrast, *B. uniformis* was more prevalent in breast-fed infants than in formula-fed infants ($P = 0.04$). Interestingly, at 4 months of age, the preva-

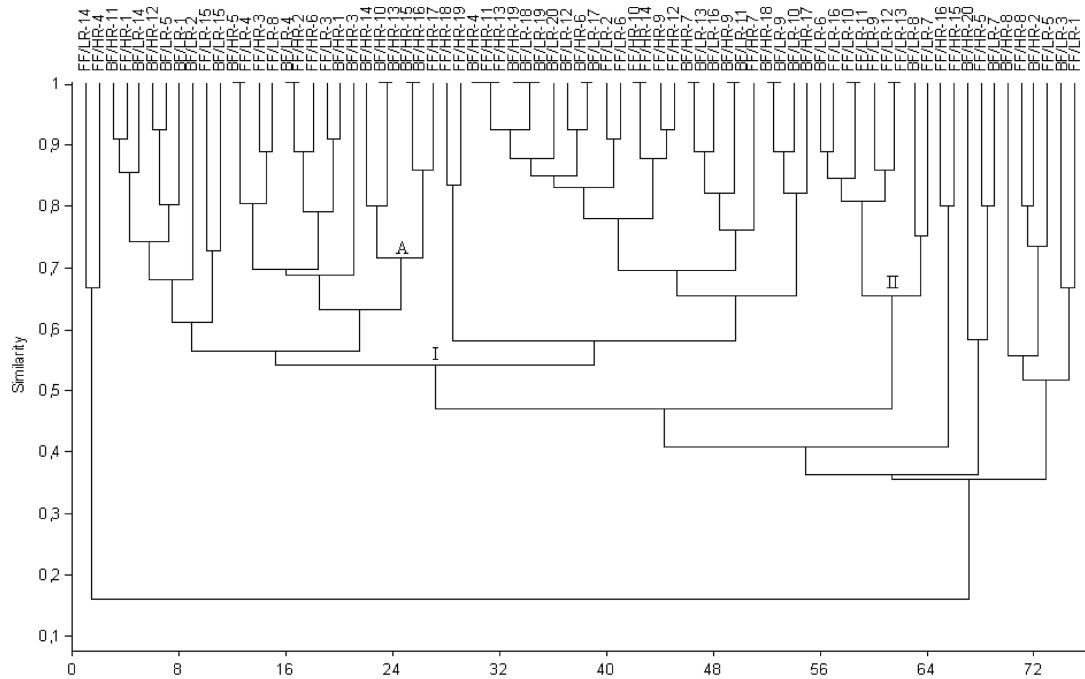


FIG. 2. Dendrogram derived from DGGE analysis of *Bacteroides* species in fecal samples of the study cohort of infants at 1 month of age, based on Dice's similarity index and the UPGMA clustering algorithm. Infants were divided according to feeding practice (breast-fed [BF] and formula-fed [FF]) and genetic risk of CD development (low risk [LR] and high risk [HR]). Letters correspond to clusters of samples.

lence of *B. uniformis* was also increased in breast-fed compared with formula-fed infants ($P = 0.04$), while that of *B. intestinalis* was higher in formula-fed infants than in breast-fed infants ($P < 0.01$). Furthermore, the prevalences of *B. caccae* and *B.*

plebeius were higher in formula-fed infants than in breast-fed infants ($P = 0.01$ and $P = 0.03$, respectively).

Influence of HLA-DQ genotype on *Bacteroides* species colonization. The diversity index of *Bacteroides* species colonizing

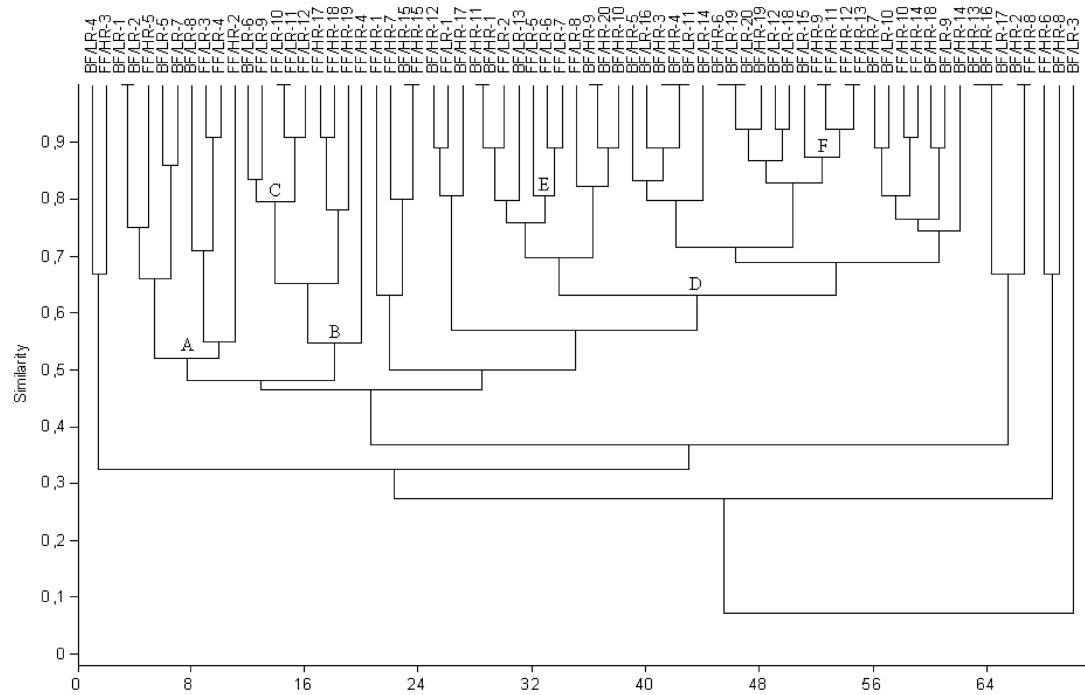


FIG. 3. Dendrogram derived from DGGE analysis of *Bacteroides* species in fecal samples of the study cohort of infants at 4 months of age, based on Dice's similarity index and the UPGMA clustering algorithm. Infants were divided according to feeding practice (breast-fed [BF] and formula-fed [FF]) and genetic risk of CD development (low risk [LR] and high risk [HR]). Letters correspond to clusters of samples.

TABLE 2. Prevalence of *Bacteroides* species detected by PCR-DGGE analysis, using Bfra531-f and Bfra766-GC-r primers and fecal DNAs from the study infants at 7 days, 1 month, and 4 months of age, stratified according to feeding practices

Species	No. (%) of infants carrying species ^a					
	7 days		1 mo		4 mo	
	Breast-fed (n = 39)	Formula-fed (n = 31)	Breast-fed (n = 40)	Formula-fed (n = 35)	Breast-fed (n = 40)	Formula-fed (n = 30)
<i>B. caccae</i>	4 (10)	12 (39)*	9 (23)	12 (34)	5 (13)	12 (40)*
<i>B. coprocola</i>	16 (41)	14 (45)	19 (48)	17 (49)	23 (58)	16 (53)
<i>B. distasonis</i>	18 (46)	15 (48)	15 (38)	20 (57)	16 (40)	14 (47)
<i>B. dorei</i>	12 (31)	6 (19)	9 (23)	8 (23)	7 (18)	5 (17)
<i>B. fragilis/B. thetaiotaomicron</i>	28 (72)	27 (87)	31 (78)	30 (86)	31 (78)	27 (90)
<i>B. intestinalis</i>	0	9 (29)*	0	4 (11)*	0	6 (20)*
<i>B. massiliensis</i>	14 (36)	5 (16)	11 (28)	7 (20)	10 (25)	5 (17)
<i>B. ovatus</i>	13 (33)	17 (55)	18 (45)	20 (57)	17 (43)	17 (57)
<i>B. plebeius^b</i>	4 (10)	8 (26)	4 (10)	9 (26)	2 (5)	7 (23)*
<i>B. uniformis</i>	24 (62)	17 (55)	27 (68)	15 (43)*	29 (73)	14 (47)*
<i>B. vulgatus</i>	33 (85)	19 (61)*	32 (80)	23 (66)	25 (63)	17 (57)

^a *, significant differences in *Bacteroides* species between breast-fed and formula-fed infants ($P < 0.05$; Fisher's exact test).

^b Species identification was done by sequencing of PCR-DGGE bands.

the infant gut, calculated by applying a linear mixed-model analysis for repeated measures, proved to be significantly higher in infants with low genetic risk than in those with high genetic risk of CD development ($P = 0.04$).

Clustering analysis of DGGE profiles obtained for infant fecal samples at 7 days and 1 and 4 months of age revealed some associations between the *Bacteroides* species profiles and the genetic risk of CD development. At 7 days of age, DGGE profiles were divided into two main groups: cluster I contained 11 profiles of infants with low genetic risk (45% similarity), and cluster II contained almost all profiles (31) for infants with high genetic risk (48% similarity) but also included one subgroup (C) containing 15 profiles of infants with low genetic risk (62% similarity) (Fig. 1). At 1 month of age, only two small clusters were detected: cluster II included 9 profiles for infants with low genetic risk (65% similarity), and cluster A included 6 profiles for infants with high genetic risk (70% similarity) (Fig. 2). At 4 months of age, 12 profiles for infants with low genetic risk were grouped into two different clusters (A and C),

with 52% and 80% similarity, respectively, while others could not be differentiated clearly from those for infants with high genetic risk (Fig. 3).

The prevalences of *Bacteroides* species in the cohort of infants, stratified according to their genetic risk of CD development, are shown in Table 3. At 7 days of age, the prevalence of *B. vulgatus* was higher in high-risk than in low-risk infants ($P < 0.01$); in contrast, *B. ovatus*, *B. plebeius*, and *B. uniformis* were more prevalent in low-risk than in high-risk infants ($P < 0.01$). The same differences were detected for these bacterial species at 1 month of age ($P = 0.03$, $P = 0.04$, $P < 0.01$, and $P < 0.01$, respectively) and 4 months of age ($P = 0.02$, $P = 0.04$, $P = 0.14$, and $P = 0.02$, respectively).

Influence of genotype on *Bacteroides* species colonizing the guts of breast-fed and formula-fed infants. The prevalences of *Bacteroides* species are shown in Table 4, presented according to the genotypes of breast-fed and formula-fed infants. Among breast-fed infants, the prevalence of *B. uniformis* was significantly higher in infants with low genetic risk than in those with

TABLE 3. Prevalence of *Bacteroides* species detected by PCR-DGGE analysis, using Bfra531-f and Bfra766-GC-r primers and fecal DNAs from the study infants with family risk of CD development at 7 days, 1 month, and 4 months of age, stratified according to genetic risk

Species	No. (%) of infants carrying species ^a					
	7 days		1 mo		4 mo	
	Low risk	High risk	Low risk	High risk	Low risk	High risk
<i>B. caccae</i>	8 (24)	8 (22)	10 (28)	11 (28)	8 (25)	9 (24)
<i>B. coprocola</i>	13 (38)	17 (47)	18 (50)	18 (46)	17 (53)	22 (58)
<i>B. distasonis</i>	16 (47)	17 (47)	19 (53)	16 (41)	14 (44)	16 (42)
<i>B. dorei</i>	7 (21)	11 (31)	7 (19)	10 (26)	4 (13)	8 (21)
<i>B. fragilis/B. thetaiotaomicron</i>	26 (76)	29 (81)	27 (75)	34 (87)	26 (81)	32 (84)
<i>B. intestinalis</i>	2 (5.9)	7 (19)	1 (2.8)	3 (7.7)	1 (3.1)	5 (13)
<i>B. massiliensis</i>	12 (35)	7 (19)	10 (28)	8 (21)	10 (31)	5 (13)
<i>B. ovatus</i>	22 (65)	8 (22)*	26 (72)	12 (31)*	21 (66)	13 (34)*
<i>B. plebeius^b</i>	10 (29)	0*	13 (36)	0*	9 (28)	0*
<i>B. uniformis</i>	24 (71)	17 (47)*	25 (69)	17 (44)*	23 (72)	20 (53)
<i>B. vulgatus</i>	20 (59)	32 (89)*	22 (61)	33 (85)*	14 (44)	28 (74)*

^a *, significant differences in *Bacteroides* species between infants with low genetic risk (<1% probability) and high genetic risk (7 to 28% probability) of developing CD ($P < 0.05$; Fisher's exact test).

^b Species identification was done by sequencing PCR-DGGE bands.

TABLE 4. Prevalence of *Bacteroides* species detected by DGGE analysis, using the primer pair Bfra531-f and Bfra766-GC-r and fecal DNAs from either breast-fed or formula-fed infants, as a function of the genetic risk of CD development (low risk, <1% probability; and high risk, 7 to 28% probability) at 7 days, 1 month, and 4 months of age

Species	Infant age	No. of breast-fed infants carrying species/total no. of infants (%)		P value ^a	No. of formula-fed infants carrying species/total no. of infants (%)		P value ^a
		Low risk	High risk		Low risk	High risk	
<i>B. caccae</i>	7 days	3/19 (15.8)	1/20 (5)	0.342	5/15 (33.3)	7/16 (43.8)	0.716
	1 mo	5/20 (25)	4/20 (20)	1	5/16 (31.3)	7/19 (36.8)	1
	4 mo	4/20 (20)	1/20 (5)	0.342	4/12 (33.3)	8/18 (44.4)	0.709
<i>B. coprocola</i>	7 days	6/19 (31.6)	10/20 (50)	0.333	7/15 (46.7)	7/16 (43.8)	1
	1 mo	10/20 (50)	9/20 (45)	1	8/16 (50)	9/19 (47.4)	1
	4 mo	10/20 (50)	13/20 (65)	0.523	7/12 (58.3)	9/18 (50)	0.722
<i>B. distasonis</i>	7 days	8/19 (42.1)	10/20 (50)	0.751	8/15 (53.3)	7/16 (43.8)	0.724
	1 mo	9/20 (45)	6/20 (30)	0.353	10/16 (62.5)	10/19 (52.6)	0.734
	4 mo	9/20 (45)	7/20 (35)	0.748	5/12 (41.7)	9/18 (50)	0.722
<i>B. dorei</i>	7 days	6/19 (31.6)	6/20 (30)	1	1/15 (6.7)	5/16 (31.3)	0.172
	1 mo	6/20 (30)	3/20 (15)	0.289	1/16 (6.3)	7/19 (36.8)	0.047*
	4 mo	4/20 (20)	3/20 (15)	1	0/12 (0)	5/18 (27.8)	0.066
<i>B. fragilis/B. thetaiotaomicron</i>	7 days	13/19 (68.4)	15/20 (75)	0.731	13/15 (86.7)	14/16 (87.5)	1
	1 mo	14/20 (70)	17/20 (85)	0.289	13/16 (81.3)	17/19 (89.5)	0.642
	4 mo	15/20 (75)	16/20 (80)	1	11/12 (91.7)	16/18 (88.9)	1
<i>B. intestinalis</i>	7 days	0/19 (0)	0/20 (0)		2/15 (13.3)	7/16 (43.8)	0.113
	1 mo	0/20 (0)	0/20 (0)		1/16 (6.3)	3/19 (15.8)	0.608
	4 mo	0/20 (0)	0/20 (0)		1/12 (8.3)	5/18 (27.8)	0.358
<i>B. massiliensis</i>	7 days	10/19 (52.6)	4/20 (20)	0.048*	2/15 (13.3)	3/16 (18.8)	1
	1 mo	8/20 (40)	3/20 (15)	0.155	2/16 (12.5)	5/19 (26.3)	0.415
	4 mo	8/20 (40)	2/20 (10)	0.065	2/12 (16.7)	3/18 (16.7)	1
<i>B. ovatus</i>	7 days	8/19 (42.1)	5/20 (25)	0.320	14/15 (93.3)	3/116 (18.8)	<0.001*
	1 mo	12/20 (60)	6/20 (30)	0.111	14/16 (87.5)	6/19 (31.6)	0.002*
	4 mo	9/20 (45)	8/20 (40)	0.762	12/12 (100)	5/18 (27.8)	<0.001*
<i>B. plebeius</i>	7 days	2/19 (10.5)	2/20 (10)	1	8/15 (53.3)	0/16 (0)	0.001*
	1 mo	4/20 (20)	0/20 (0)	0.106	9/16 (56.3)	0/19 (0)	<0.001*
	4 mo	2/20 (10)	0/20 (0)	0.244	7/12 (58.3)	0/18 (0)	0.002*
<i>B. uniformis</i>	7 days	16/19 (84.2)	8/20 (40)	0.008*	8/15 (53.3)	9/16 (56.3)	1
	1 mo	18/20 (90)	9/20 (45)	0.019*	7/16 (43.8)	8/19 (42.1)	1
	4 mo	17/20 (85)	12/20 (60)	0.155	6/12 (50)	8/18 (44.4)	1
<i>B. vulgatus</i>	7 days	15/19 (78.9)	18/20 (90)	0.695	5/15 (33.3)	14/16 (87.5)	0.003*
	1 mo	15/20 (75)	17/20 (85)	0.695	7/16 (43.8)	16/19 (84.2)	0.030*
	4 mo	11/20 (55)	14/20 (70)	0.353	3/12 (25)	14/18 (77.8)	0.008*

^a *, statistical differences between the genetic risk groups. Statistical differences were calculated by using Fisher's exact test and considering differences significant if the P value was <0.05.

high genetic risk during the study period, but significant differences were detected only at 7 days and 1 month of age ($P < 0.01$ and $P = 0.02$, respectively). Among formula-fed infants, the prevalence rates of *B. ovatus* and *B. plebeius* were higher ($P = <0.001$ to 0.002) in infants with low genetic risk than in those with high genetic risk of CD development, while the prevalence of *B. vulgatus* was higher in infants with high genetic risk ($P = 0.003$ to 0.030).

DISCUSSION

The present study provides the first evidence of the effects exerted by both milk-feeding practices and CD genetic risk

factors on the intestinal colonization process of *Bacteroides* species in infants at early life.

Our study indicates that milk-feeding practices influence both *Bacteroides* diversity and species prevalence. This study reports an increased prevalence of *B. vulgatus* in breast-fed infants at 7 days of age, which is not in agreement with the results of the only published related study, which reported that *B. vulgatus* and *B. thetaiotaomicron* were predominant in fecal samples from 5- to 6-day-old formula-supplemented newborns (23).

Genetic predisposition to CD has been linked to the MHC region on chromosome 6p21, with over 95% of CD patients expressing HLA-DQ2 or -DQ8 (26). In our study,

infants were classified into two groups according to HLA-DQ genotype, with one including those with a high probability of developing CD (7 to 28%) and the other including those with a low probability (<1%) of developing the disease (5). Infants with high genetic risk had an increased prevalence of *B. vulgatus* and a reduced prevalence of *B. ovatus*, *B. plebeius*, and *B. uniformis* compared with infants with low genetic risk. Enterocytes that are in close proximity with the intestinal contents and bacteria can express HLA-II molecules of the MHC to a certain extent and are able to act as antigen-presenting cells (16). Moreover, HLA-II molecules are expressed primarily by dendritic cells present in the lamina propria, which can extend dendrites across this epithelium, capture bacteria and other antigens at the mucosal surface, and present the antigens to B or T lymphocytes (6). This process is a critical step in the initiation of a mucosal immune response, and its possible role in restricting bacterial colonization cannot be disregarded (14). In fact, the polysaccharide of *B. fragilis* has been proven to associate with MHC-II molecules with a high affinity, through a mechanism mirroring peptide presentation, which could mediate MHC-II antigen presentation and, ultimately, T cell recognition during bacterial infection (7).

In a preliminary study of exclusively breast-fed infants, larger proportions of the *Bacteroides-Prevotella* group were detected in infants with high genetic risk than in those with low genetic risk of CD development (8); however, the effect of milk-feeding type was not considered. The present study provides a wider characterization of the *Bacteroides* population at the species level and also takes into account the variable of milk-feeding type. In this study, we demonstrated that the prevalence of *B. vulgatus* was increased in infants with high genetic risk, considering both the total cohort and formula-fed infants only. Furthermore, in the whole cohort of infants, prevalence rates for *B. uniformis*, *B. ovatus*, and *B. plebeius* were also increased in the group with low genetic risk, and this trend was confirmed by considering the subgroup of either breast-fed or formula-fed infants. These results indicate that the HLA-DQ genotype has an independent effect on the colonization of these species, in addition to that exerted by the type of milk feeding, which could also influence the disease risk.

The colonization process of the intestine shortly after birth seems to be important for adequate immune response maturation and to the risk of suffering immune-mediated diseases. A few studies suggest that *B. fragilis* could be involved in this process, influencing Toll-like receptor 4 (TLR4) mRNA in peripheral blood monocytes (27) or the number of circulating IgA and IgM antibody-producing cells (11, 19). *In vitro* studies also indicate that exposure to polysaccharide A of *B. fragilis* activates CD4⁺ T cells, resulting in a Th1 response, which could modify the risk of suffering CD (29). Nevertheless, neither *in vitro* nor *in vivo* information is available regarding the potential immunological role of other *Bacteroides* species.

In summary, the results indicate that both the milk-feeding type and the HLA-DQ genotype influence the colonization process of *Bacteroides* species early in life. An increased *B. vulgatus* prevalence was associated with the genotype of

infants at high risk of CD development, while increased *B. uniformis* prevalence was associated with the genotype of infants at low risk of CD development and with breast-feeding, which could partly explain the protective effect of breast-feeding. Further research is under way to disclose a possible relationship between these findings and subsequent development of CD in a follow-up study of this cohort of infants.

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