Oligosaccharides in 4 Different Milk Groups, *Bifidobacteria*, and *Ruminococcus obeum*

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ABSTRACT

Objectives: The aim was of this study is to identify a link between the total amount of breast milk oligosaccharides and faecal microbiota composition of newborns at the end of the first month of life, with special attention paid to bifidobacteria, and establish the role, if any, of the different oligosaccharides in determining the gut microbiota composition.

Subjects and Methods: Milk oligosaccharide groups were identified by high-performance anion exchange chromatography analysis. DPCRNA from newborns' faecal samples at 30 days of life was isolated and processed by polymerase chain reaction analyses that allow the identification of 6 species of bifidobacteria (*adolescentis*, *bifidum*, *breve*, *catenulatum*, *longum*, *infantis*) and *Ruminococcus* spp; denaturing gradient gel electrophoresis analysis was also performed.

Results: No substantial differences in bifidobacteria species composition within milk groups 1, 2, and 3 were observed; however, infants fed with group 4 milk show a microbiota characterised by a greater frequency of *Bifidobacteria adolescentis* and the absence of *Bifidobacteria catenulatum*. For the first time, a high percentage of the *Ruminococcus* genus in infants fed with all milk groups was found.

Conclusions: Our data show that milk groups 1, 2, and 3, containing an amount of oligosaccharides ranging within 10 to 15 g/L, share a substantially identical composition of the intestinal microbiota in breast-fed infants, despite quali-quantitative difference in oligosaccharides content. Newborns taking milk with only 5 g/L of oligosaccharides (group 4) harbour a different intestinal microbiota.

Key Words: *Bifidobacterium*, breast-fed infants, human milk oligosaccharides, intestinal microbiota, *Ruminococcus*

(*JPGN* 2011;00: 00–00)

Under normal conditions, the human gastrointestinal tract at birth is sterile. Initially, the development of intestinal microbiota is modulated by several extrinsic factors such as the type of delivery, the environmental contamination, the sanitary conditions, and the geographical distribution of bacterial species (1–3). Afterwards, the main factor conditioning the development of the newborn's intestinal ecosystem is represented by the kind of feeding (4). In fact, after using the most recent microbiological methods (5), the intestinal microbiota of the breast-fed infant is represented up to 90% by bifidobacteria and lactobacilli (6), whereas in the bottle-fed newborn these 2 genera represent only 40% to 60% of the intestinal bacteria, and the remaining is represented by *Enterobacteriaceae* and *Bacteroides* (6–8). The several beneficial effects of a microbiota dominated by bifidobacteria in breast-fed infants are well known and have been emphasised by the Committee on Nutrition of the European Society of Paediatric Gastroenterology, Hepatology, and Nutrition (9).

Some studies carried out with molecular methods on breast-fed newborns' faeces have recently shown the presence of the genus *Ruminococcus*, described as an important component of a baby's intestinal microbiota (10,11) because it produces ruminococcin A, a bacteriocin that can inhibit the development of a lot of species of *Clostridium* (12,13).

Several substances in human milk such as phosphorus and nucleotides (14–17), can promote the development of the bifidobacteria-dominant microbiota, but for most of them only sporadic and discordant data are reported; however, well documented is the positive effect of oligosaccharides in stimulating the development of the bifidobacteria (18). A high percentage of such substances resist digestion in the gastrointestinal tract, reach the colon, and stimulate the microbiota development, representing the paradigm of the prebiotics (19–23).

On the basis of their chemical composition, oligosaccharides can be classified as follows: core oligosaccharides, made of glucose (Glc), galactose (Gal), and *N*-acetyl-glucosamine (GlcNAc), representing the starting structures for the synthesis of more complex oligosaccharides; fucosyl-oligosaccharides, derived from the addition of fucose (Fuc) to lactose and other core structures; and sialyl-oligosaccharides, ensuing from the addition of sialic acid (NANA) to lactose and core oligosaccharides.

The presence of the various glycosyltransferases in the mammary gland is genetically determined. Most of them are common to all women, the exception being fucosyltransferases, whose presence is linked to the expression of the secretor (Se) and Lewis (Le) genes in the mammary gland (24).

Depending on the expression of Se and Le genes both the qualitative and the quantitative compositions of milk oligosaccharides are significantly conditioned. With reference to the presence or absence of specific fucosyl-oligosaccharides, 4 different groups of human milk have been recognised by Thurl et al (25): group 1 milk (Se^+/Le^+; about 70% of the European population): containing all types of fucosyl-oligosaccharides, with α1,2 to α1,3 and α1,4 linkages; group 2 milk (Se^-/Le^+; 20% of the population): without α1,2 fucosyl-oligosaccharides, as the result of the lack of expression of the Se gene; group 3 milk (Se^+/Le^-; 9% of the general population): characterised by the absence of α1,4 fucosyl-oligo-

Received April 12, 2010; accepted November 16, 2010.

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The authors report no conflicts of interest.

DOI: 10.1097/MPG.0b013e3182073103

saccharides because of the inactivity of the Le gene; group 4 milk (Se^-/Le^-; 1% of the general population): containing only α1,3 fucosyl-oligosaccharides resulting from the expression of the Le independent fucosyltranferase.

From the above data it appears that the majority of the results on the intestinal microbiota of breast-fed infants reported in the literature to date likely reflects the effect of milk group 1, at least in Europe. For this reason the aim of the present pilot study was essentially focused to investigate whether the different qualitative and quantitative oligosaccharide content of the more rare milk groups could play some effects on intestinal microbiota composition, with special attention paid to bifidobacterial and ruminococcal populations.

SUBJECTS AND METHODS

Subjects and Sample Collection

The research was approved by the hospital ethics committees and written informed consent was obtained from all of the mothers before their participation in the study. Healthy mothers delivering full-term normal newborns vaginally were selected. A morning sample (between 6 and 9 AM) was obtained by manual expression into sterile plastic tubes after the infant had suckled for at least 5 minutes (mid-feed sampling technique) (26). No significant differences in the concentrations of oligosaccharide and lactose during the feeding have been reported (27,28). Milk samples were immediately frozen and stored at −20°C until analysis.

At the 3rd to 5th day postpartum, samples of milk from 256 nursing mothers were collected and qualitatively analysed by thin-layer chromatography (TLC) as previously described (22). On the basis of their oligosaccharide pattern, samples were assigned to 1 of the 4 groups of Thurl et al (25). Successively, 10 mothers belonging to group 1 milk were randomly selected together with all groups 2, 3, and 4 mothers identified. A total of 39 mothers and their babies were recalled between the 25th and the 35th day after delivery to collect a second sample of milk and a sample of newborn faeces; 10 mothers belonged to group 1, 19 to group 2, 6 to group 3, and 4 to group 4. Newborns or mothers receiving antibiotic therapy were excluded from the study. All of the milk and faecal samples were maintained at −20°C until use.

Qualitative and Quantitative Characterisation of Human Milk

Oligosaccharides of milk samples selected at the end of the first month were analysed by high-performance anion exchange chromatography (HPAEC), as previously described (29). Briefly, 1 mL acetonitrile was added to the same amount of milk; after stirring and centrifugation, the supernatant was diluted to 1:24 (vol/vol) with water and filtered through a 0.22-μm membrane. A 25-μL sample was injected by autosampler in a Dionex high-performance liquid chromatography A1 450 system consisting of a CarboPac PA pre-column, a CarboPac PA-1 column, and a PAD II detector (Dionex, Sunnyvale, CA). Separation was obtained using as eluent 1 a 100-mmol/L NaOH solution and as eluent 2 a 100-mmol/L NaOH solution with 1 mol/L Na acetate. The system was eluted at the flow rate of 0.7 mL/min as previously described in detail. The respective amount of the different oligosaccharides was automatically calculated on calibration curves obtained using 3 different concentrations of single pure standards obtained from Sigma Chemical Co (St Louis, MO), Bio Carb (Lund, Sweden), and Dextra Laboratories Ltd (Reading, UK). The following 18 main oligosaccharides (from a quantitative point of view) were measured: lacto-*N*-tetraose, lacto-*N*-neotetraose, lacto-*N*-hexaose, and lacto-*N*-neohexaose (core oligosaccharides); lacto-*N*-difuco-hexaose II, trifucosyl-lacto-*N*-hexaose, difucosyllacto-*N*-hexaose, 3-fucosyllactose, lacto-*N*-fucopentaose II, 2′-fucosyllactose, lacto-N-fucopentaose I, monofucosyllacto-*N*-hexaose II (fucosyl-oligosaccharides); and sialyl-lacto-*N*-neotetraose c, 6′-sialyllactose, sialyllacto-*N*-tetraose a, 3′-sialyllactose, sialyllacto-*N*-tetraose b, disialyllacto-*N*-tetraose (sialyl-oligosaccharides). HPAEC allowed us to confirm precisely the assignment of each milk sample to 1 of the 4 groups on the basis of the presence or the absence of 2′-fucosyllactose and lacto-*N*-fucopentaose II, markers of the expression of the Se and Le genes, respectively.

DNA Extraction

DNA was extracted by using 1 g of each frozen faecal sample, homogenised in 2 mL of extraction buffer (100 mmol/L Tris-HCl pH 8, 10 mmol/L EDTA, 25% sucrose, and 5 mg/mL of lysozyme) and incubated for 1 h at 37°C. Then the suspension was centrifuged at 10,000 rpm for 10 minutes; the pellet obtained was further resuspended in 0.7 mL of 20 mmol/L EDTA and 70 μL of 10% SDS solution was added. After the addition of 40 μL of proteinase K solution (20 mg/mL), the sample was incubated for 1 to 2 hours at 55°C and then 160 μL of sodium perchlorate 6 mol/L was added. The supernatant was further extracted with phenol-chloroform, treated with DNase-free RNase, and precipitated with ethanol.

PCR Amplifications

Primers used in this study, either as a preliminary step for denaturing gradient gel electrophoresis (DGGE) analysis or as species-specific identification tools, are available in the literature, and for this reason, their sequences have not been reported in the present article.

Primers described by Matsuki et al (30) have been used to identify the 6 species more frequently recovered from infant faeces: *Bifidobacterium adolescentis*, *Bifidobacterium catenulatum* group, *B infantis*, *B longum*, *B breve*, and *B bifidum.* To detect the presence of the *Ruminococcus obeum* group we used primers suggested by Wang et al (31) with some modifications to the original amplification cycle (94°C for 2 minutes, then 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds, 68°C for 1 minute, and finally 68°C for 7 minutes).

To obtain a polymerase chain reaction (PCR) product suitable for DGGE analysis, representing the V2-V3 region of the 16S rRNA gene, HDA1-GC and HDA2 primers were used. DGGE analysis of PCR amplicons was performed using the DCode or D-GENE System apparatus (Bio-Rad, Hercules, CA).

DGGE was performed as described by Walter et al (32) with the following modifications: the gel contained a 35% to 55% gradient of urea and formamide increasing in the direction of the electrophoresis, as indicated in a further publication of Dal Bello et al (33). In the PCR thermal cycle we used a melting time of 1 minute and a number of cycles raised to 35. For the amplification required to perform the DGGE specific for the *Bifidobacterium* genus a pair of primers, Bif662-r and Bif164-GC-f, was used as described by Satokari et al (34). For the amplicons obtained polyacrylamide gels (8% [wt/vol] acrylamide-bisacrylamide [37.5:1]) in 0.5× Tris-acetic acid-EDTA buffer with a denaturing gradient were prepared with a gradient mixer and Econopump (Bio-Rad) using solutions containing 45% and 55% denaturant. A 100% denaturant corresponds to 7 mol/L urea and 40% (vol/vol) formamide. PCR amplicons were separated by electrophoresis at a

constant voltage of 85 V and at a temperature of 60°C for 16 hours. The gels were stained with SybrGreen (Roche, Mannheim, Germany). Excision and purification of DNA fragments from the gels have been carried out as described by Ben Omar and Ampe (35) and then reamplified with the original primers under the conditions described. PCR products obtained from the reamplification were purified using the Wizard SV Gel and PCR clean-up system (Promega, Madison, WI). Final eluants containing purified PCR fragments were sequenced at BMR Bio Molecular Research (CRIBI, Università di Padova, Italy). The sequences obtained were compared with those available in public databases by using BLAST analysis of sequences from the Ribosomal Database Project (36).

RESULTS

Characterisation of Oligosaccharides

In Table 1 the content of 8 fucosyl-oligosaccharides in the 4 different groups of the studied nursing mothers is reported. Milk from the women of group 1 contains all of the analysed fucosyl-oligosaccharides. In groups 2 and 3 the total amount of fucosyl-oligosaccharides is about 35% and 45% compared with group 1 because of the lack of Se-dependent α1,2 fucosyl-oligosaccharides in group 2 and of α1,3/4 Le-dependent compounds in group 3. Nursing mothers' milk from group 4 has a slight quantity of fucosyl-oligosaccharides corresponding to about 8% of the milk of the subjects of group 1 because of the presence of α1,3 Le-independent fucosyl-oligosaccharides only. As regards the content of core and sialyl-oligosaccharides, no qualitative differences were observed in the 4 groups because all of the analysed oligosaccharides are present in each group. From the quantitative point of view, the core oligosaccharides altogether had an average concentration (expressed as mean ± SD) of 2.21 ± 0.17 g/L in group 1, 1.86 ± 0.15 in group 2, 2.48 ± 0.21 in group 3, and 2.30 ± 0.22 in group 4; the sialyl-oligosaccharides have an average concentration of about 2 g/L (group 1: 2.11 ± 0.12 g/L; group 2: 1.90 ± 0.10 g/L; group 3: 2.02 ± 0.12 g/L; group 4: 1.96 ± 0.09 g/L). As a result, the whole content of core and sialyl-oligosaccharides in each group is about 4 g/L. In consequence, the total content of human milk oligosaccharides is strictly dependent on the presence or absence of the fucosyl-oligosaccharides, and it shows a wide variation in mature milk ranging from about 15 g/L in the subjects of group 1 to about 5 g/L in the subjects of group 4. Intermediate values (about 8–9 g/L) are present in the subjects of groups 2 and 3. Figure 1 clearly shows the differences in the oligosaccharide patterns within the 4 milk groups.

Microbiological Assessment

Group 1

Ten subjects fed on this type of milk have been considered. DGGE analysis by means of universal primers has emphasised the presence of some dominant bands but without a common, distinctive pattern among the different samples. Two bands emerged as common in all of the profiles; both of them, after sequencing, evidenced homology (96%) with *B breve* GeneBank sequences. One of these bands has been detected in 9 of 10 samples within the group, whereas the second common band has been detected in 6 of 10 samples. No further common bands in DGGE patterns have been detected. The presence of *Bifidobacterium* species has been also checked by genus-specific DGGE (Fig. 2) and confirmed by multiplex PCR. Both techniques confirmed that the dominant species is *B breve* (detected in 7 of 10 by DGGE and 6 of 10 with multiplex), followed by *B longum* (detected in 3 samples of 10 by both techniques), and *B bifidum* (evidenced by *Bifidobacterium*-specific DGGE in 4 of 10 samples, but in only 3 of these 4 samples by multiplex PCR) (Table 2). We established that the number of bifidobacterial species for this babies' group ranged from 0 to 3 for each sample if considered singularly. For what concerns the detection of *R obeum*, in 4 samples a significant PCR signal has been evidenced on the agarose gel for group 1 (Table 2).

Group 2

Nineteen babies have been analysed; universal DGGE profiles evidenced a common pattern of 2 bands (Fig. 3). The first was present in 10 samples of 19; sequence analysis showed 97% of similarity with *B longum*. The second band, detected in 13 of 19 samples, was found to be homologous (94% DNA homology) to *Escherichia coli*. Within this group multiplex PCR has evidenced the presence of 3 dominant bifidobacteria: *B catenulatum*, *B longum*, and *B breve*. In Table 2, the distribution of the 3 species within the samples belonging to the group is shown. The results obtained by species-specific PCR have been partially confirmed by the DGGE technique specific for *Bifidobacterium*, which has in their case also produced data sometimes in accord and other times different from those obtained with the other 2 applied techniques. Some examples of the DGGE profiles obtained with bifido-specific primers are reported in Figure 2. By PCR species-specific *B catenulatum* has been detected in 15 of 19 samples; *B longum*–positive signal appeared in 10 of 19 samples; and *B breve* has been detected in 12 of the 19 collected samples. These results have been partially confirmed by *Bifidobacterium*-specific DGGE. In fact, *B catenulatum* was differentiated by specific DGGE in only 12

TABLE 1. Fucosyl-oligosaccharides in different milk groups

	α Linkages	LNDFH II	TFLNH	DFLNH	3 FL	LNFP II	2′ FL	LNFP I	MFLNH II	Total
Group 1, n = 10	1–2 1–3 1–4	0.20 ± 0.06	2.95 ± 1.2	2.01 ± 0.61	0.36 ± 0.12	0.45 ± 0.13	2.56 ± 0.90	1.18 ± 0.30	0.45 ± 0.15	10.16
Group 2, n = 19	1–3 1–4	0.23 ± 0.05	nd	2.10 ± 0.56	0.40 ± 0.09	0.48 ± 0.10	nd	nd	0.42 ± 0.08	3.63
Group 3, n = 6	1–2 1–3	nd	nd	nd	0.41 ± 0.10	nd	2.66 ± 0.85	1.25 ± 0.32	0.43 ± 0.09	4.75
Group 4, n = 4	1–3	nd	nd	nd	0.44 ± 0.11	nd	nd	nd	0.38 ± 0.12	0.82

Values are expressed as grams per liter (mean ± SD); 2′ FL = 2′-fucosyllactose; 3 FL = 3-fucosyllactose; DFLNH = difucosyllacto-*N*-hexaose; LNDFH II = lacto-*N*-difuco-hexaose II; LNFP I = lacto-*N*-fucopentaose I; LNFP II = lacto-*N*-fucopentaose II; MFLNH II = monofucosyllacto-*N*-hexaose II; nd = not detected; TFLNH = trifucosyllacto-*N*-hexaose.

samples of the 15 evidenced by multiplex PCR as positive. The same occurred in 1 case for *B breve*, found to be present by specific multiplex PCR but absent in the DGGE profile.

These bacteria have been detected with species-specific PCR in most of the samples, which ranged from 1 to 4 species in each. *R obeum* has revealed a massive presence (15 of 19 samples analysed) in this group by PCR signal obtained with species-specific primers.

Group 3

Six individuals have been considered in this group. DGGE performed with universal primers has evidenced only 2 bands present in at least 50% of samples. The first band has been found in 5 of 6 profiles and appeared to correspond to *B longum* (94% homology). The latter band, detected in 3 of 6 samples, after sequencing was found to match (98%) with *B bifidum*. Among the subjects belonging to this group, the 3 different techniques used to detect bifidobacteria evidenced some discrepancies (Table 2). Multiplex PCR showed the presence of *B longum* in 4 of 6 samples considered, whereas the bifido-specific DGGE evidenced a band corresponding to this species (99% DNA homology) in only 3 of these samples. As regards the presence of *R obeum* within this group, the specific amplification has been detected in 50% of the samples.

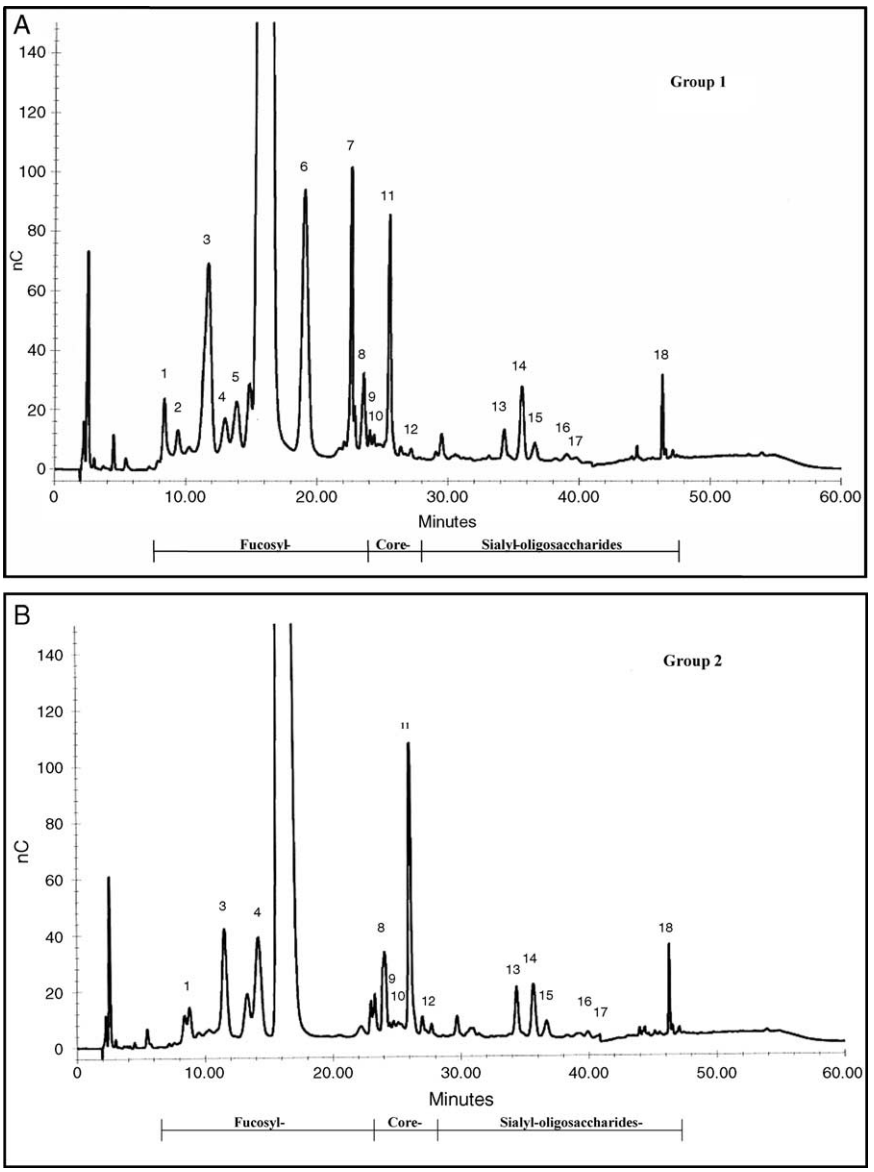

FIGURE 1. High-performance anion exchange chromatography of oligosaccharides from human milk groups 1–4. Peak 1: lacto-*N*-difuco-hexaose II; peak 2: trifucosyllacto-*N*-hexaose; peak 3: difucosyllacto-*N*-hehaose; peak 4: 3-fucosyllactose difucosyllacto-*N*-hexaose; peak 5: lacto-*N*-fucopentaose II; peak 6: 2′-fucosyllactose; peak 7; lacto-*N*-fucopentaose I; peak 8: monofucosyllacto-*N*-hexaose II; peak 9: lacto-*N*-neotetraose; peak 10: lacto-*N*-neohexaose; peak 11: lacto-*N*-tetraose; peak 12: lacto-*N*-hexaose; peak 13: sialylacto-*N*-neotetraose c; peak 14: 6′-sialyllactose; peak 15: sialyllacto-*N*-tetraose a; peak 16: 3′-sialyllactose; peak 17: sialyllacto-*N*-tetraose b; peak 18: disialyllacto-*N*-tetraose.

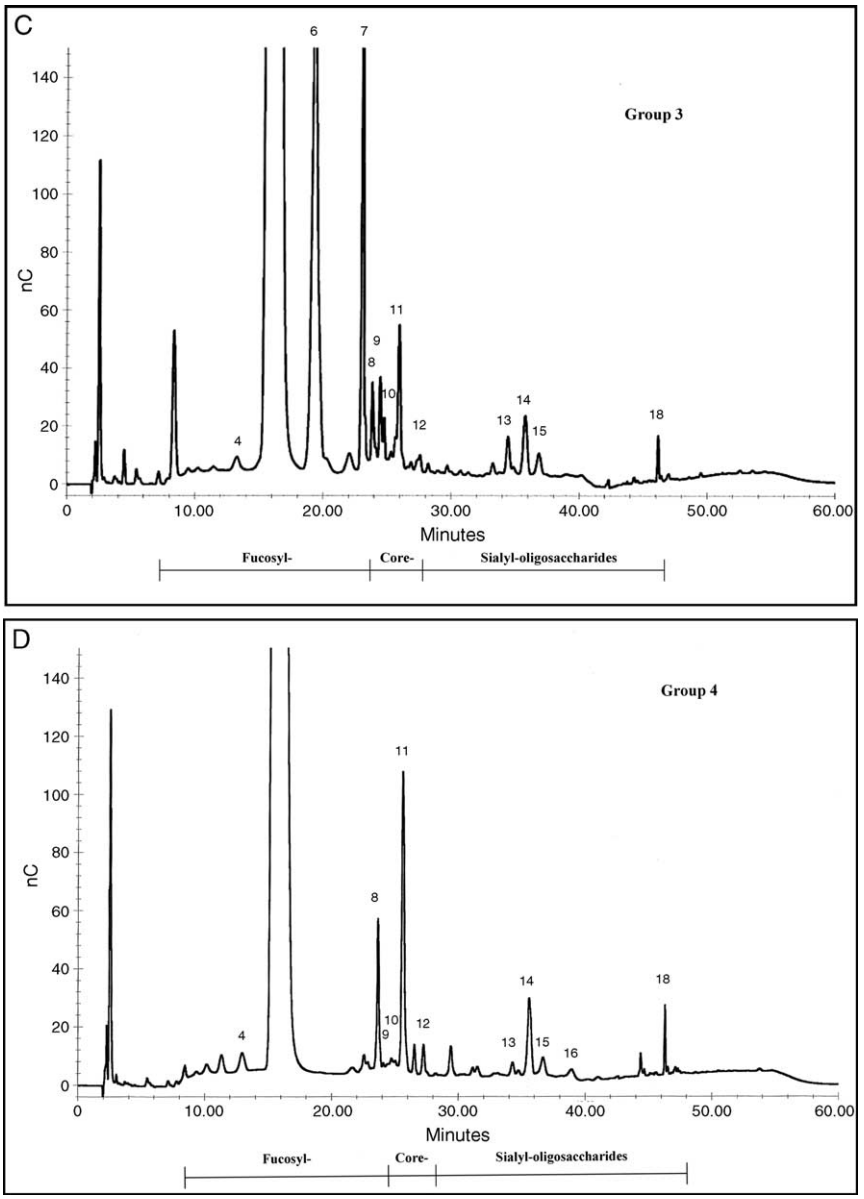

FIGURE 1. (*continued*).

Group 4

DGGE results obtained performing universal primers' amplification on the 4 subjects considered in this group did not evidence a common pattern (Fig. 4), except for 1 band present in 75% of the subjects belonging to the group. This has revealed, after sequencing, a similar sequence corresponding to *B breve* (97%). A second band was present in 50% of samples and was revealed to be homologous (98%) to *B bifidum*.

Multiplex PCR and *Bifidobacterium*-specific DGGE also confirmed these results because both techniques gave positive signals in 3 of 4 samples for *B breve* and in 2 of the 4 samples analysed for *B bifidum* (Table 2). Moreover, *B catenulatum* was not detected in any sample and *B adolescentis* was present in 50% of the subjects of this group. The presence of *R obeum* in this group was shown for the 75% of the samples collected.

DISCUSSION

Numerous studies of microbial colonisation of the human intestine at birth have been performed, and the fluctuation of the faecal populations of newborns has been well established (1,10,11,37).

The techniques of traditional microbiology have shown some limitations in representing the real composition of such a complex and mainly anaerobic microbiota as the intestinal one. That is why to study the population of the intestinal tract of children of paediatric age we have used molecular techniques such as PCR-DGGE and multiplex specific PCR, which belong to the ''culture-independent techniques'' able to show the presence of genera, which normal laboratory practice makes difficult to cultivate.

As documented by other authors, the overall intestinal microbiota pattern is characteristic of each individual, but at the

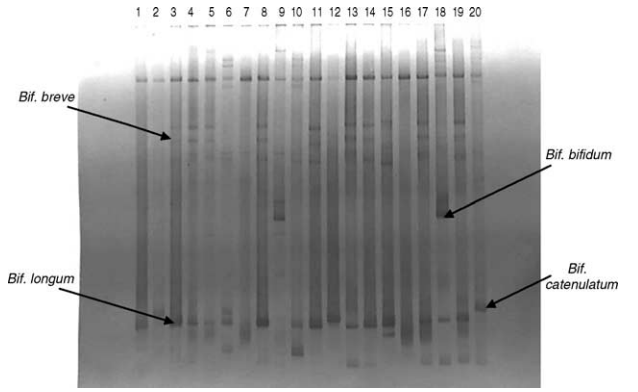

FIGURE 2. *Bifidobacterium*-specific polymerase chain reaction-denaturing gradient gel electrophoresis obtained from faecal samples of babies belonging to different groups. The arrows indicate the most frequent *Bifidobacterium* species identified by fragment sequencing for babies of the 4 groups. Lanes 8, 9, 12, 17, and 20 are referred to samples belonging to group 1. Lanes 1–3, 5, 6, 10, 11, 13–16, and 18 are referred to group 2. Lane 19 represents the profile of a baby belonging to group 3 and in lanes 4 and 7, we report samples of group 4.

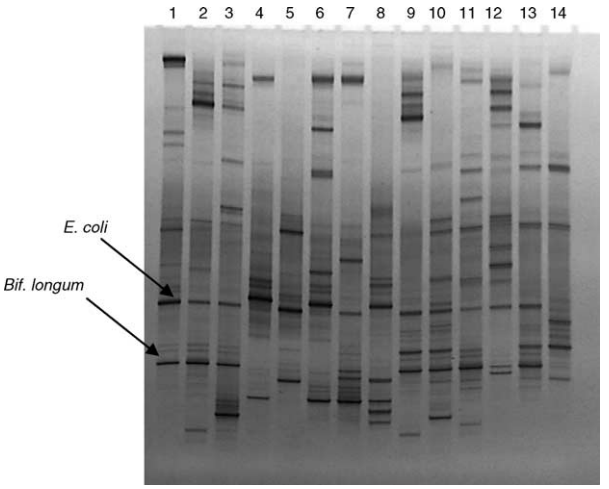

FIGURE 3. Denaturing gradient gel electrophoresis of polymerase chain reaction products of V2–V3 region of 16S rDNA of some faecal samples from babies belonging to group 2. The arrows indicate the 2 common bands among the profile belonging to this group.

same time some bacterial genera are universally present and relatively stable in time (5,38,39). It is also known that human milk is able to promote the development of a specific intestinal microbiota that has positive effects on the newborn's health. It is also known that among the different components of milk, oligosaccharides play the most important role in the development of the intestinal microbiota. Moreover, the qualitative and quantitative content of oligosaccharides presents wide differences that have led to the identification of 4 groups with remarkably different prevalence on the general population (25). Our data show that the total oligosaccharide content of human milk ranges from about 15.0 g/L in group 1 to about 5.0 g/L in group 4 because of the presence or absence of specific fucosyl-oligosaccharides.

As far as we know, there are no studies available about a possible relation between the content of oligosaccharides in these 4 human milk groups and the composition of the intestinal microbiota. Considering that the nursing mothers of group 1 represent about 70% of the general population, it is likely that the data reported in the literature on the newborns' microbiota mainly concern the newborns fed by the mothers of this group.

The results we obtained after the analysis of individuals of the 4 milk groups, even with numerical differences between the single groups, allow us to make considerations. The first is the absence of a relation between the type of milk oligosaccharides and the presence of *B breve*, which resulted in the most common *Bifidobacterium* species inhabiting the intestinal tract of all of the breast-fed babies considered in our study (28 of 39 samples). This finding is partially in accordance with several studies (40,41) that have shown that certain bifidobacterial species (*B infantis*, *B breve*, *B longum*) were often detected in the intestinal flora of breast-fed and formula-fed infants. The regular presence of *B infantis* was not confirmed by other studies (42), and in our study this bacterium was detected in only 1 of 39 samples. The second dominant species we found was *B longum* (present in 18 of 39 samples), a species frequently isolated from human adult microbiota (43).

Concerning the number of species belonging to the genus *Bifidobacterium*, the present study shows that the different composition in fucosyl-oligosaccharides of groups 1, 2, and 3 milks does not seem to have a particular influence on intestinal microbiota of breast-fed infants. On the contrary, intestinal microbiota of newborns fed with group 4 milk, containing <1.0 g/L of fucosyl-oligosaccharides, did not show any colonisation *by B catenulatum*. In the same group, *B adolescentis*, a species more commonly found in adults than in infants (30), was found in a higher percentage compared with the other 3 groups. A possible explanation for these

TABLE 2. Positive results (%) obtained for each group from DGGE and PCR species-specific analyses

Bifidobacterium spp	Group 1 (10 subjects)		Group 2 (19 subjects)		Group 3 (6 subjects)		Group 4 (4 subjects)	
	DGGE	PCR	DGGE	PCR	DGGE	PCR	DGGE	PCR
B breve	70	60	68	63	83	66	75	75
B catenulatum	20	40	63	79	33	50	—	—
B longum	30	30	53	53	50	66	25	25
B bifidum	40	30	21	32	50	66	50	50
B adolescentis	10	10	10	10	—	—	50	50
B infantis	—	—	5	5	—	—	—	—
R obeum	I 40		I 79		50		75	

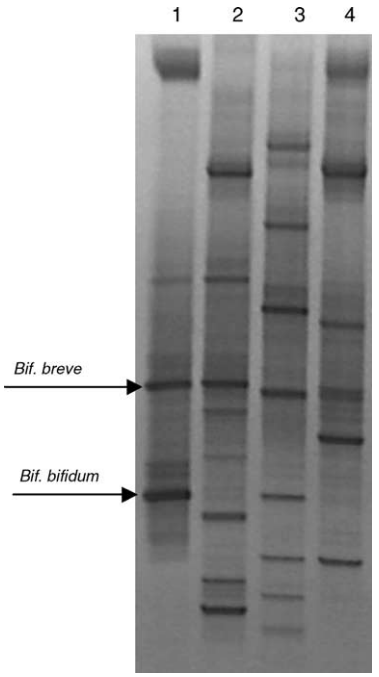

FIGURE 4. Polymerase chain reaction-denaturing gradient gel electrophoresis profiles generated by HDA1-HDA2 amplification from faecal samples obtained from the 4 babies belonging to group 4. The arrows indicate the only 2 distinctive tracts found in the group.

findings could be related to the low content of fucosyl-oligosaccharides because no substantial differences exist within the 4 groups of milk concerning both sialyl- and core oligosaccharides. Of course, because of the low number of subjects studied, it is not possible to draw definitive conclusions; however, these results clearly suggest that further investigations could be worthwhile. Considering that the groups differ in their composition in fucosyl-oligosaccharides, it can be assumed that the fucosydic residues in different positions of the oligosaccharide molecules are not the only cause for the prebiotic effect of human milk. In fact, the core and sialyl-oligosaccharides present in almost the same quantities in all 4 milk groups could contribute to the development of intestinal microbiota.

The presence of *E coli* as 1 of the most important colonisers in intestinal infant microbiota has been reported in many studies (10,11,44,45). Vaginally delivered infants harbour *E coli* more frequently as compared with caesarean section–delivered infants, whereas the latter more frequently carried other enterobacteria, such as *Klebsiella* and *Enterobacter*. Independent of delivery mode, it took 2 months until most infants were colonised by enterobacteria, traditionally the first colonizers.

An additional interesting result is the detection in all of the groups studied and in more than half of the subjects of the *Ruminococcus* genus, already reported to be present in newborns by Favier et al (10), who studied 3 infants. Our data suggest a possible major role played by ruminococci as dominant bacteria, at the same level as bifidobacteria, in the gut of breast-fed babies (10,46). *Ruminococcus* genus is an anaerobe close to the filogenetic group of *Clostridium coccoides* (47) and it has been studied since 1965 as a cellulase producer in ruminants (48). Further studies have proved that ruminococci represent an important component of the intestinal microbiota in adults. Less information is reported about the incidence of *Ruminococcus* on the infant's intestinal microbiota,

TABLE 3. Percentage of finding of the different species of *Bifidobacterium* tested on all 39 subjects analysed

Bifidobacterium species	DGGE	PCR
B catenulatum	41	56
B adolescentis	13	13
B infantis	3	3
B longum	44	46
B breve	72	72
B bifidum	33	38
R obeum	—	64

in particular in breast-fed babies compared with formula-fed ones. Almost no information is available about human milk components that exert specific prebiotic effects to promote the development of such bacteria.

The enzymatic activities allowing some species of bifidobacteria or ruminococci to degrade the mucin molecules and human milk oligosaccharides (19) may explain the simultaneous presence of these 2 genera (49,50). Our data suggest that strains of *Ruminococcus* have similar levels of presence of those species of *Bifidobacterium* found to be the most represented in breast-fed babies analysed in the present study. In particular, *R obeum* is present at the same level as *B breve* and *B catenulatum* for the majority of the investigated faecal samples (Table 3). This observation could open the path to future investigations on the possible role played by *Ruminococcus* in the health of breast-fed infants.

The last comment is a technical one, considering that there is an effective difference in sensibility shown by the 2 *Bifidobacterium*-specific DGGE and multiplex species-specific PCR techniques in identifying the presence of species belonging to this genus in the samples. Specific DGGE detection of bifidobacterial species in faeces was not as sensitive as species-specific PCR, with the exception of only a few cases. In all of the other cases we speculate that only bifidobacterial species whose cells were the most numerous were detected in faeces with Bif 164-f and Bif 662-GC-r primers and subsequent DGGE.

In conclusion, our data suggest that the quali-quantitative differences in fucosyl-oligosaccharide content in groups 1, 2, and 3 milks stimulate colonisation of rather similar gut microbiota composition in all breast-fed babies. Our preliminary results in infants fed with group 4 milk show a microbiota characterised by a greater frequency of *B adolescentis* and the absence of *B catenulatum* when compared with infants fed with other group milks.

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