The Journal of Nutrition
Influence of Diet on Infection and Allergy in Infants

Cesarean Delivery May Affect the Early Biodiversity of Intestinal Bacteria[1,2]

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Abstract

The gastrointestinal tract of neonates becomes colonized immediately after birth with environmental microorganisms, mainly from the mother; strong evidence suggests that the early composition of the microbiota of neonates plays an important role for the postnatal development of the immune system. The present study was designed to evaluate by means of a molecular biology approach the relation between the intestinal ecosystem of the newborn and the mode of delivery. The intestinal bacterial composition on d 3 of life was investigated in 23 infants born by vaginal delivery and in 23 infants delivered by cesarean section. PCR-denaturing gradient gel electrophoresis and PCR-temperature gradient gel electrophoresis have been utilized, together with the specific amplifications for 10 *Bifidobacterium* species, 3 *Ruminococcus* species, and *Bacteroides*. The intestinal microbiota of neonates delivered by cesarean delivery appears to be less diverse, in terms of bacteria species, than the microbiota of vaginally delivered infants. The intestinal microbiota after cesarean delivery is characterized by an absence of *Bifidobacteria* species. Vaginally delivered neonates, even if they showed individual microbial profiles, were characterized by predominant groups such as *B. longum* and *B. catenulatum*. Our data demonstrate that the mode of delivery has a deep impact on the composition of the intestinal microbiota at the very beginning of human life. This study opens the path to further investigations to confirm the link between microbiota composition and immune system development and to identify tools for the modulation of the intestinal microbiota of cesarean-delivered neonates. Additionally, we underline the importance of adequate microbiological tools used to support clinically relevant trials, if intestinal microbiota is considered as a study outcome. J. Nutr. 138: 1796S–1800S, 2008.

Introduction

The intestinal microbiota composition of the neonate has not been clearly defined yet, as many bacterial species living in the gut are unculturable under laboratory conditions. An alternative approach for microbial identification is provided by culture-independent techniques such as the exploitation, by means of molecular biology techniques, of polymorphisms of genes encoding for bacterial 16S rRNA (1). These techniques have been applied to evaluate the gut microbiota composition of adults and infants, but a lack of knowledge still exists on the comparison of gut microbiota of neonates delivered by cesarean section to the microbiota of naturally delivered babies.

The gastrointestinal tract of newborns is sterile, but it becomes colonized immediately after birth with organisms from the environment, mainly from the mother. During vaginal delivery, the contact with the vaginal and intestinal flora is an important source for the start of the infant's colonization (2). During cesarean delivery, direct contact of the mouth of the newborn with the vaginal and intestinal microbiota is absent, and environmental bacteria play an important role for infants' intestinal colonization. Some authors have suggested that the composition of the very first human microbiota could have long-lasting effects, up to months (3) or even years (4).

There is accumulating evidence that intestinal bacteria play an important role in the postnatal development of the immune system (5,6). Thus, if the intestinal flora develops differently depending on the mode of delivery, the postnatal development of the immune system might also be different. Available epidemiological data show that atopic diseases appear more often in infants after cesarean delivery than after vaginal delivery (7–10). The composition of enteric microbiota in early days of life seems therefore to be a very important factor for achieving and maintaining good health in the years to come. It follows that it is fundamental to identify more thoroughly the intestinal ecosystem of the newborn.

[1] Published as a supplement to *The Journal of Nutrition*. Presented at the symposium "Infant Nutrition" held in Rotterdam, The Netherlands, September 8, 2006. The symposium was organized by the Sophia Children's Hospital, Erasmus University, Rotterdam, The Netherlands, and was cosponsored by Danone Research Wageningen, The Netherlands. Supplement coordinators: G. Boehm and J. B. van Goudoever, Erasmus University, The Netherlands. Supplement coordinator disclosures: G. Boehm is an employee of Danone Research, the sponsor of the supplement; J. B. van Goudoever, no relationships to disclose.

[2] Author disclosures: G. Boehm is an employee of Danone Research, the sponsor of the supplement; G. Basucci, B. Benenati, L. Morelli, and E. Bessi, no conflicts of interest.

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TABLE 1 Clinical characteristics of subjects enrolled[1]

	Vaginal delivery	Cesarean delivery
n (male/female)	23 (11/12)	23 (8/15)
Gestational age, *wk*	39.6 ± 1.3 (38–41)	39.4 ± 1.3 (37–42)
Birth weight, *g*	3366 ± 487 (2480–4070)	3389 ± 576 (2620–5110)
Birth length, *cm*	50.2 ± 1.6 (47–52)	49.9 ± 2.4 (46–55)
Head circumference, *cm*	34.6 ± 1.0 (33–36.5)	35.1 ± 1.4 (33–37.5)
Exclusively breast fed,[2] *n*	20	21

[1] Values are means ± SD (range) or *n*.
[2] Data refer to the number of newborns with already detected amount of breast milk ingested within the first 3 d of life. None of the babies enrolled received infant formula.

In this study, we investigated the influence of mode of delivery (cesarean delivery vs. vaginal delivery) on the intestinal microbial composition on d 3 of life using PCR-denaturing gradient gel electrophoresis (DGGE) and PCR-temperature gradient gel electrophoresis (TGGE). Both DGGE and TGGE analyses have been utilized, together with the specific amplifications for 10 *Bifidobacterium* species, 3 *Ruminococcus* species, and *Bacteroides*, all playing a very relevant physiological role in the intestinal ecosystem of the newborn (11).

Subjects, Materials, and Methods

Forty-six term infants born in October 2003 at the Guglielmo da Saliceto Hospital, Piacenza, Italy were eligible for the study (**Table 1**).

The study protocol was approved by the local Medical Ethical Committee. In this study, written informed consent was obtained from parents of all the enrolled newborns, whether born by vaginal or cesarean delivery. The study was designed to consecutively recruit all the eligible cesarean and the immediately following spontaneously delivered babies. Exclusion criteria were prematurity, maternal infections during pregnancy, maternal clinical illness, maternal specific dietary regimen (i.e., vegetarian or any exclusion diet for any reason) as reported by means of last-week dietary recall, maternal antibiotic administration, or probiotic supplementation during the last 2 wk of gestation, intrapartum antibiotic prophylaxis, and babies given antibiotic prophylaxis or therapy. All babies enrolled were of Caucasian origin; none of the babies enrolled were fed infant formula; and all were breast-fed on demand. The fecal samples were obtained on d 3 of life, before the infants' discharge from the hospital and were stored at –20°C in sterile containers until the analysis.

DNA analysis

Bacterial DNA was extracted using PSP Spin Stool DNA kit (Invitek, Berlin).

The differentiation of the amplicons obtained through the amplification of bacterial DNA was achieved by DGGE and TGGE techniques.

DGGE and TGGE analysis with PCR amplification. To amplify V6-V8 regions on bacterial DNA, we utilized U968-GC-f and L1401-r primers (11). To amplify specific regions for *Bifidobacterium* spp., primers Bif164-f and Bif662-GC-r were used (12).

DGGE analysis of amplified samples. The fragments obtained by PCR as reported were separated through DGGE (Biorad) as described by Favier et al. (11), using a 40–50% gradient for separating fragments obtained by amplification of regions V6-V8 and a 45–55% gradient for fragments obtained with primers for *Bifidobacterium*. The electropho-

TABLE 2 Species-specific primers used to detect microbial flora in fecal samples

Primers	Target	Amplified weight, *bp*
BiADO-1 (5′- CTC CAG TTG GAT GCA TGT C -3′)	*Bifidobacterium adolescentis*	279
BiADO-2 (5′- CGA AGG CTT GCT CCC AGT -3′)		
BiANG-1 (5′- CAG TCC ATC GCA TGG TGG T -3′)	*B. angulatum*	275
BiANG-2 (5′- GAA GGC TTG CTC CCC AAC -3′)		
BiBIF-1 (5′- CCA CAT GAT CGC ATG TGA TTG -3′)	*B. bifidum*	278
BiBIF-2 (5′- CCG AAG GCT TGC TCC CAA A -3′)		
BiBRE-1 (5′- CCG GAT GCT CCA TCA CAC -3′)	*B. breve*	288
BiBRE-2 (5′- ACA AAG TGC CTT GCT CCC T -3′)		
BiCATg-1 (5′- CGG ATG CTC CGA CTC CT -3′)	*B. catenulatum* (group)	289
BiCATg-2 (5′- CGA AGG CTT GCT CCC GAT -3′)		
BiDENT-1 (5′- ATC CCG GGG GTT CGC CCT -3′)	*B. dentium*	387
BiDENT-2 (5′- GAA GGG CTT GCT CCC GA -3′)		
BiGAL-1 (5′- TAA TACC CGG ATG TTC CGC TC -3′)	*B. gallicum*	303
BiGAL-2 (5′- ACA TCC CCG AAA GGA CGC -3′)		
BiINF-1 (5′- TTC CAG TTG ATC GCA TGG TC -3′)	*B. infantis*	828
BiINF-2 (5′- GGA AAC CCC ATC TCT GGG AT -3′)		
BifLAC-2 (5′- GTG GAG ACA CGG TTT CCC -3′)	*B. lactis*	680
BifLAC-5 (5′- CAC ACC ACA CAA TCC AAT AC -3′)		
BiLONG-1 (5′- TTC CAG TTG ATC GCA TGG TC -3′)	*B. longum*	831
BiLONG-2 (5′- GGG AAG CCG TAT CTC TAC GA -3′)		
BFR-1 (5′- ACTCTTTGTATCCCGACGATT -3′)	*Bacteroides fragilis* (group)	582
BFR-2 (5′- GAGGTTGATGCCTGTATCGGT -3′)		342
BFR-3 (5′- GCTACCGAAGTATGCCAGATTACA -3′)		
BFR-4 (5′- GTGGTCATTCGCCAGATTACA -3′)		
ROB-3 (5′- TGA GGA GAC TGC CAG GGA -3′)	*Ruminococcus obeum* (group)	312
ROB-2 (5′- CTC CTT CTT TGC AGT TAG GT -3′)		
RCA-1 (5′- CGC ATA ACA TCA TGG ATT CG -3′)	*R. callidus*	286
RCA-2 (5′- CGT CAT TAT CGT CCT CTT CA -3′)		
RBR-5 (5′- GAA GTA GAG ATA CAT TAG GTG -3′)	*R. bromii*	444
RBR-6 (5′- ACG AGG TTG GAC TAC TGA -3′)		

retic run was carried out for 16 h at 85 V. DNA fragments, separated by DGGE, were extracted from the electrophoretic gel and identified by sequencing and successive alignment with sequences of the GenBank (www.ncbi.nlm.nih.gov/BLAST/) database (13).

TGGE analysis of amplified samples. The fragments coding for regions V6-V8 on bacterial DNA obtained by PCR have been separated by TGGE as described by Satokari et al. (14), with a 37°C to 46°C temperature gradient, 0.5 ramp, and an 18-h run at 85 V.

PCR amplification with species-specific primers. Some microbial species considered to be very important in the neonatal intestinal microbiota have been investigated by PCR with species-specific primers (**Table 2**) (14–16).

The presence of bacteria belonging to the genus *Bifidobacterium*, with the exception of *B. catenulatum* and *B. lactis*, was also investigated by species-specific multiplex PCR. Species-specific amplification of *B. lactis* has been carried out according to Ventura et al. (17). *Bacteroides fragilis* group, *R. obeum* group, *R. callidus* e *R. bromii* PCR-based detection was carried out according to Yamashita et al. (15) and Wang et al. (16).

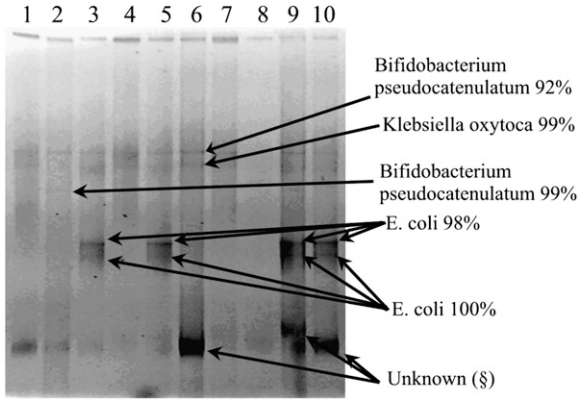

FIGURE 2 DGGE analysis of PCR product, with U968-r and L1401-f universal primers, of fecal samples from vaginal-delivery babies (§). These bands were not sequenced because, after amplification, they did not produce any analyzable products.

Results and Discussion

DGGE profiles obtained by means of universal primers on fecal samples of newborns delivered either by cesarean section or vaginally were usually characterized by few bands, most of which were in common with all the other subjects in each of the 2 groups considered (**Fig. 1**, **Fig. 2**). In particular, bands corresponding to *Klebsiella oxytoca* and *Bifidobacterium pseudolongum* seem to be present in all the lines obtained from the feces of vaginally delivered infants.

Slight differences have been found, on the other hand, with respect to *E. coli* (Fig. 2). This microbial group, in fact, was found in 9 of 23 (39.1%) spontaneously delivered newborns, whereas overlapping bands were founds only in 2 of 23 (8.7%) cesarean-delivered newborns (Fig. 1).

DGGE analysis, carried out with *Bifidobacterium*-specific primers, revealed the presence of this genus in 13 of 23 (56.5%) samples derived from vaginally delivered newborns but in none of the samples obtained from newborns delivered by cesarean section.

TGGE analysis, carried out with universal primers on fecal samples of both groups of subjects, showed greater inter- and intragroup profile variations, particularly in the group of babies born by vaginal delivery (**Fig. 3**). In contrast, those born by cesarean section displayed more constant TGGE profiles (**Fig. 4**).

PCR analysis with *Bifidobacterium* species-specific primers showed that naturally delivered infants had a large number of bifidobacterial species, whereas in cesarean-delivered babies, only 2 samples (8.7%) gave positive results, 1 for *B. longum* and the other for *B. gallicum*.

With regard to the qualitative pattern, spontaneously delivered babies show greater differences concerning investigated *Bifidobacterium* species; the most represented are *Bifidobacterium catenulatum* group and *Bifidobacterium longum*; *B. breve* was detected in 52.2% of samples, *B. bifidum* in 39.1%; *B. infantis*, *B. gallicum*, and *B. adolescentis* species were more scarce (17.4, 4.3, and 21.7%, respectively). In all babies enrolled, microorganisms belonging to *Ruminococcus* species are absent, and *Bacteroides* has been found in 8.7% of spontaneously delivered babies only.

Species-specific PCR analysis shows the presence of *B. catenulatum* and *B. longum* in 43.5% of vaginally delivered babies, sometimes accompanied by the presence of *B. breve*, *B. bifidum*, or *B. adolescentis*.

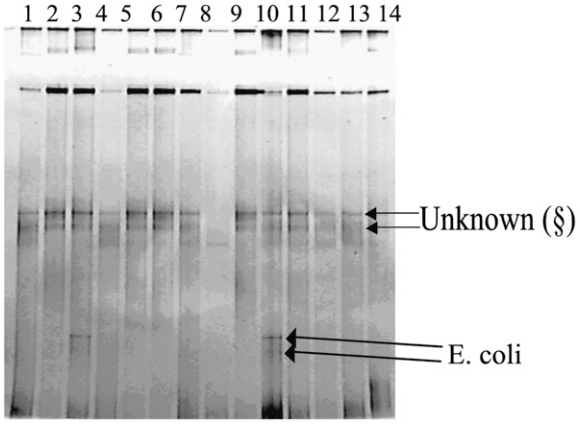

FIGURE 1 DGGE analysis of PCR product, with U968-r and L1401-f universal primers, of fecal samples from cesarean-delivery babies. (§) These bands were not sequenced because, after amplification, they did not produce any analyzable products.

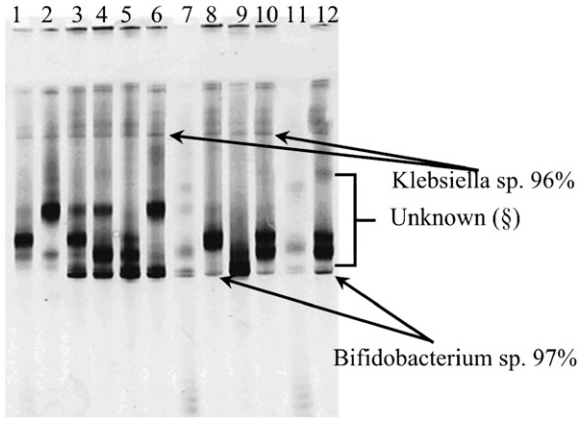

FIGURE 3 TGGE analysis of PCR product, with U968-r and L1401-f universal primers, of fecal samples from vaginal-delivery babies (§). These bands were not sequenced because, after amplification, they did not produce any analyzable products.

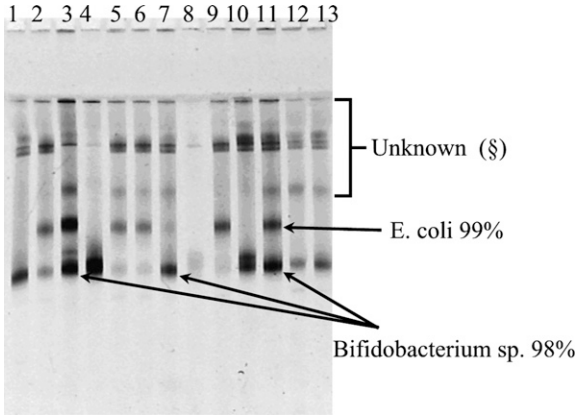

FIGURE 4 TGGE analysis of PCR product, with U968-r and L1401-f universal primers, of fecal samples from cesarean-delivery (§). These bands were not sequenced because, after amplification, they did not produce any analyzable products.

Specifically, in this group of newborns, 17.4% are colonized at the same time by *B. breve*, *B. bifidum*, the *B. catenulatum* group, and *B. longum*, whereas *B. breve*, the *B. catenulatum* group, and *B. longum* are present in 8.7% of babies.

Although some studies (11) demonstrate that bifidobacteria appear after d 2 or 3 of life and usually dominate after the first 2 wk of life, because of feeding-related differences in the colonization time, little is known concerning the first 3 d after birth. Our study demonstrates that the newborn's intestinal microbiota, with respect to presence of bacteria, is strongly influenced, within 3 d of life, by the mode of delivery. From a qualitative point of view, the intestinal flora of cesarean- and vaginally delivered infants appears to be very different. The intestinal flora of infants by cesarean delivery is characterized by a substantial absence of *Bifidobacteria* species. Infants by vaginal delivery show subject-specific microbial profiles, although predominant groups such as *B. longum* and *B. catenulatum* group could be identified.

If the intestinal colonization could have health effects, the clear difference in the intestinal flora between vaginally delivered and cesarean-delivered infants would be of clinical relevance. Because only 2 infants born by cesarean delivery had not yet received a detectable amount of breast milk within 3 d of life, at least at this early age, the kind of nutrition seems to have a less crucial impact in modulating intestinal microbiota composition. In addition, we point out the great importance of an adequate microbiological approach in this kind of study.

DGGE analysis seems to be less sensitive than species-specific PCR analysis because the latter has provided the identification of a larger number of *Bifidobacterium* species not detected by DGGE. TGGE, on the other hand, seems to have a greater discrimination power than DGGE and to allow more differentiated profiles with respect to individual intestinal microbiota.

There is an increasing body of evidence that the intestinal microbiota could play an essential role in the postnatal development of the immune system (18,19). Malamitsi-Puchner et al. (20) found that only vaginal delivery promotes the production of various cytokines implicated in neonatal immunity. Hällström et al. (21) found a link among mode of cesarean delivery, disturbed intestinal colonization, and, possibly, occurrence of necrotizing enterocolitis in preterm infants.

Although the link between mode of delivery and clinical outcome is multifactorial, there is also evidence that the circumstances of a cesarean delivery have influence on the later outcome. In epidemiological studies, it could be demonstrated that elective cesarean delivery provides an increased risk for allergic diseases in later childhood (7–10). The particular role of the starting intestinal flora for long-term effects was emphasized by all investigators (7–10).

Data available from several studies indicate a delayed onset of lactation with cesarean section (22–24). Thus, many infants who were born by cesarean delivery also lacked the early support of breast milk as stimulator for a physiological intestinal flora. Both, the nonphysiological start of colonization and the missing early dietary support by delayed start of lactation might result in these long-lasting effects.

In summary, our data indicate that the early stage of the bacterial intestinal colonization in infants born by cesarean delivery is altered from that of infants born vaginally, with no or little influence of the type of feeding. Additionally, our results emphasize the importance of the methodological aspects for determining intestinal microbiota in clinical trials, if intestinal microbiota composition is to be considered a measure of postnatal adaptation.

Other articles in this supplement include references (25–34).

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