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Fecal Microflora in Healthy Infants Born by Different Methods of Delivery: Permanent Changes in Intestinal Flora After Cesarean Delivery

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ABSTRACT
Background: Newborn infants in modern maternity hospitals are subject to numerous factors that affect normal intestinal colonization—for example, cesarean delivery and antimicrobial agents. To study the duration of the effect of external factors on intestinal colonization, two groups of infants with different delivery methods were investigated.
Methods: The fecal flora of 64 healthy infants was studied prospectively. Thirty-four infants were delivered vaginally, and 30 by cesarean birth with antibiotic prophylaxis administered to their mothers before the delivery. The fecal flora was cultured on nonselective and selective media in infants 3 to 5, 10, 30, 60, and 180 days of age. Gastrointestinal signs were recorded daily by the mothers for 2 months.
Results: The fecal colonization of infants born by cesarean delivery was delayed. *Bifidobacterium*-like bacteria and *Lactobacillus*-like bacteria colonization rates reached the rates of vaginally delivered infants at 1 month and 10 days, respectively. Infants born by cesarean delivery were significantly less often colonized with bacteria of the *Bacteroides fragilis* group than were vaginally delivered infants: At 6 months the rates were 36% and 76%, respectively ($p = 0.009$). The occurrence of gastrointestinal signs did not differ between the study groups.
Conclusions: This study shows for the first time that the primary gut flora in infants born by cesarean delivery may be disturbed for up to 6 months after the birth. The clinical relevance of these changes is unknown, and even longer follow-up is needed to establish how long-lasting these alterations of the primary gut flora can be. ***JPGN 28:19-25, 1999.*** **Key Words:** Bacteroides—Cesarean delivery—Colic—Colonization—Gut—Intestine.

The neonatal period is crucial for intestinal colonization. Born sterile and undergoing colonization within a few days, infants are an open field for colonization by different types of bacteria. Gestational age, type of delivery, and feeding affect the stool flora of young infants (1), but there is little information on which long-term factors influence the bacterial selection process in the gut of young infants (2).

Infants born vaginally apparently acquire their gut flora from maternal vaginal and fecal flora (3), but the environment also contributes. Within maternity wards, nosocomial spread is shown to exist among healthy newborn infants (4,5). For the colonization of infants born by cesarean delivery (CD), the environment is extremely important (6,7). Likewise, if infants are separated from their mothers for long periods after birth, the environment becomes an important source of colonizing bacteria (5).

Gut colonization is delayed in infants born by CD, and intestinal colonization is consequently abnormal for several weeks (1,8). Most of the studies on gut colonization of infants born by CD have extended only to the first month of life (1,8–10). Overall, however, the long-term stability of the gut flora of newborn infants has been studied systematically in only a few studies (2,11). Such information would clearly be valuable, because changes in the primary gut flora have been associated with gastrointestinal disorders and infantile colic (12–15). We studied the stability of the changes in the fecal flora of newborn infants and the association between certain bacteria and gastrointestinal signs. To study two distinctly different groups of neonates with different gut flora, two study groups were formed: vaginally delivered (VD) infants ($n = 34$) and infants born by CD to mothers who had received antimicrobial prophylaxis ($n = 30$). The fecal flora was recorded for 6 months, and gastrointestinal signs were registered daily for 2 months.

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MATERIALS AND METHODS

Infants

The study included 64 healthy newborn infants of healthy mothers who delivered at the Department of Obstetrics and Gynecology, University Central Hospital, Turku, Finland. Thirty-four VD infants and 30 infants delivered by elective CD were enrolled between February 1995 and June 1996 after written informed consent had been obtained from their parents. The enrollment rate was two to four children per week, because sampling necessitated that the birth took place on Monday through Thursday. The mothers who delivered by cesarean birth received prophylactically 2 g intravenous ampicillin 2 hours before the operation. None of the mothers had received any antimicrobial agents within the month that preceded the delivery. After delivery, the newborns were admitted randomly to one of the two maternity wards for healthy newborn infants.

Fecal Samples and Bacterial Cultures

Fecal samples were taken when the infants were 3 to 5 days old (taken at the hospital) and 10, 30, 60, and 180 days old (taken at home). The specimens were collected in plastic containers. If not cultured immediately, the samples were stored at 4°C. The specimens obtained at home were taken to the laboratory by the parents. The mean storage time of the specimens was 10.7 hours (range, 0.5–34.5 hours).

An approximate 300-mg portion of the specimen was weighed, diluted, and homogenized in fastidious anaerobe broth (Lab M, Bury, UK). Serial 100-fold dilutions were made in the same broth. Duplicate samples of 10 μl of each dilution were cultured on a variety of nonselective and selective media (Table 1).

The MacConkey plates were incubated at 35°C in ambient air for 24 hours. All other cultures were incubated in anaerobic jars with gas-generating kits (Anero Gen, AN35; Oxoid, Basingstoke, UK) at 35°C for 48 hours (fastidious anaerobe agar [FAA], *Bacteroides* bile esculin agar [BBE] and *Clostridium perfringens* selective agar), or for 72 hours (Rogosa and modified Petuely's agars). The number of colonies from two parallel plates was counted from a dilution yielding 30 to 300 colony-forming units (CFU)/plate, and the average was recorded.

The total number of colonies was counted and recorded from MacConkey and FAA agars. The number of all different types of colonies was counted from BBE, Rogosa, and modified Petuely's agars and the number of distinctive black colonies from *Clostridium perfringens* agar. All different types of colonies from BBE, Rogosa, and modified Petuely's agars and distinctive black colonies from *Clostridium perfringens* agar were subcultured for aerotolerance testing and were Gram stained.

Anaerobic gram-negative, esculin hydrolysing rods growing on BBE agar were recorded as bacteria of the *Bacteroides fragilis* group. Anaerobic and aerotolerant nonbranching, gram-positive rods with parallel sides from Rogosa agar were recorded as *Lactobacillus*-like bacteria (LLB). It has been shown that all colonies growing well on Rogosa agar may be considered lactic acid bacteria; some enterococci and pediococci may show reduced growth (16). Anaerobic and aerotolerant gram-positive rods from modified Petuely's agars were recorded as *Bifidobacterium*-like bacteria (BLB). Modified Petuely's agar is highly selective and efficient for detection of *Bifidobacterium* from fecal samples. In a previous work by Tanaka and Mutai (17) 175 fecal strains were detected from the modified Petuely's medium; from these 94% were bifidobacteria, 3% eubacteria, and 3% peptostreptococci.

Clostridium perfringens was identified by its distinct colony and Gram-stain morphology, by its anaerobic nature in aerotolerance testing, and by positive results in the reversed CAMP test (18). The bacterial counts were expressed as the $\log_{10}$ of colony-forming units per gram of wet weight of feces.

Clinical Symptoms

The mothers kept a diary for 2 months of their infants' daily bowel habits, abdominal distension, flatulence, normal and colicky crying, use of antimicrobial agents, and feedings. Crying was defined as colicky if it was a distinctive pain cry, and the infant was difficult to console (19). Abdominal distension and flatulence were given a daily score by the following scale: no signs, a few signs, moderate signs, or heavy signs.

TABLE 1. *Culture media and dilutions*

Medium	Bacteria	Dilutions
MacConkey agar (CM7, Oxoid, Basingstoke, UK)	Aerobic enteric bacteria[a]	3-day, 10-day, 30-day samples[b] 10^5–10^9 60-day, 180-day samples 10^7–10^{11}
Fastidious anerobe agar (Lab M, Bury, UK)	Total count[c]	3-day, 10-day, 30-day samples 10^5–10^9 60-day, 180-day samples 10^7–10^{11}
Bacteroides bile esculin agar (Reference 21)	*Bacteroides fragilis* group	3-day, 10-day, 30-day samples 10^3–10^7 60-day, 180-day samples 10^5–10^9
Clostridium perfringens selective agar (CM 543, Oxoid)	*Clostridium perfringens*	3-day, 10-day, 30-day samples 10^3–10^7 60-day, 180-day samples 10^5–10^9
Rogosa agar (CM 627, Oxoid)	*Lactobacillus*-like bacteria[d]	3-day sample 10^3–10^7 10-day, 30-day, 60-day, 180-day samples 10^5–10^9
Modified Petuely's agar (Reference 17)	*Bifidobacterium*-like bacteria	3-day sample 10^3–10^7 10-day, 30-day, 60-day, 180-day samples 10^5–10^9

[a] Includes enterobacteria, staphylococci, enterococci.
[b] 3–5-day, 10-day, and 30-day samples.
[c] Includes facultative and anaerobic bacteria.
[d] Includes gram-positive, non-branching rods with parallel sides.

Statistical Analysis

The results were analysed statistically by Fisher's exact test (to compare the number of infants colonized at each time point), Mann–Whitney's rank sum test (to compare bacteria counts of colonized infants at 3 days and 6 months of age), and analysis of variance of repeated measurements (to compare the gastrointestinal signs). A commercial software program (Statistica, version 5.0; Stat Soft, Tulsa, OK, U.S.A.) was used for these calculations. $P < 0.05$ was considered statistically significant.

ETHICAL CONSIDERATIONS

The study was approved by the joint Committee of Ethics of the Turku University and Turku University Central Hospital.

RESULTS

The study groups differed slightly by gestational age (VD group, 40 weeks; CD group, 39 weeks; $p = 0.04$) but the birth weights did not differ statistically (VD group, 3577 g; CD group, 3572 g; $p = 0.96$). Special attention was paid to the method of feeding the infants. The use of formula was recorded carefully by the mothers. The proportion of infants exclusively breast-fed at 2 months of age or partly breast-fed at 6 months of age did not differ between the study groups ($p = 0.2$ and 0.8, respectively). Eleven infants received antimicrobial agents during follow-up. All of these therapies were administered when the infants were older than 2 months, and in these infants the subsequent 6-month fecal samples were excluded from the analysis. (Table 2)

TABLE 2. *Study subjects*

	Vaginally delivered ($n = 34$)	Cesarean delivery ($n = 30$)	p
Mean birth weight in grams (range)	3577 (2850–4480)	3572 (2640–4320)	0.96[a]
Mean gestational age in weeks (range)	40 (37–42)	39 (37–42)	0.04[a]
Number of boys	16	12	0.6[b]
Number of infants exclusively breast-fed for 2 months	19	22	0.2[b]
Number of infants partly breast-fed for 6 months[c]	17	13	0.8[b]
Number of infants receiving antibiotic regimen during follow-up	3	8	0.1[b]

[a] Two-sample t-test.
[b] Fisher's exact test.
[c] Information missing from two vaginally delivered infants and from three cesarean births.

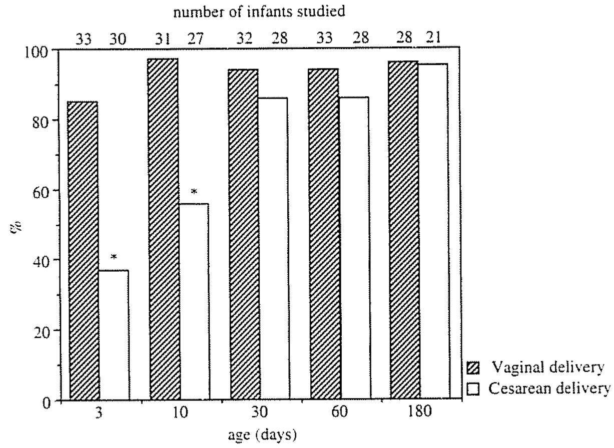

FIG. 1. The percentage of *Bifidobacterium*-like bacteria (BLB) colonization in infants aged 3, 10, 30, 60, and 180 days born vaginally and by cesarean delivery. *$p < 0.001$.

Colonization Rates

With the exception of one infant (in the CD group) who was culture-negative at 3 days of age, all infants were colonized with aerobic enteric bacteria in every culture. The colonization rates of BLB and LLB were lower in the CD group than in the VD group after birth. The colonization rate of BLB coincided in these groups by 1 month and of LLB by 10 days of age (Figs. 1 and 2). The colonization rate of LLB in the CD group even exceeded that of the VD group in infants 2 and 6 months of age (Fig. 2). The *Clostridium perfringens* colonization rate was statistically higher in the CD group than in the VD group at 1 month of age (57% vs. 17%; $p = 0.003$; Fig. 3).

The colonization rates of bacteria within the *Bacteroides fragilis* group differed most markedly of all between the study groups. The *Bacteroides* colonization rate ranged from 52% to 79% in the VD group (Fig. 4). Only one of the infants in the CD group was *Bacteroides*-positive at 3 days of age. After that, *Bacteroides* was not recovered in any of the samples from the infants in the CD group before the age of 2 months. In infants 6 months of age, the colonization rate was still statistically lower in the CD group than in the VD group (36% vs. 76%; $p = 0.009$; Fig. 4).

Colonization Levels

The colonization levels of the different bacteria of the colonized infants are shown in Table 3. The total bacterial counts were significantly lower in the CD group in infants 3 days of age ($p = 0.005$) and in those 6 months of age ($p = 0.03$). The aerobic enteric bacterial counts did not differ between the groups. Infants in the CD group had a significantly lower level of BLB when they were 3 days old ($p = 0.005$) but not when they were 6 months old ($p = 0.5$). There were no differences in the

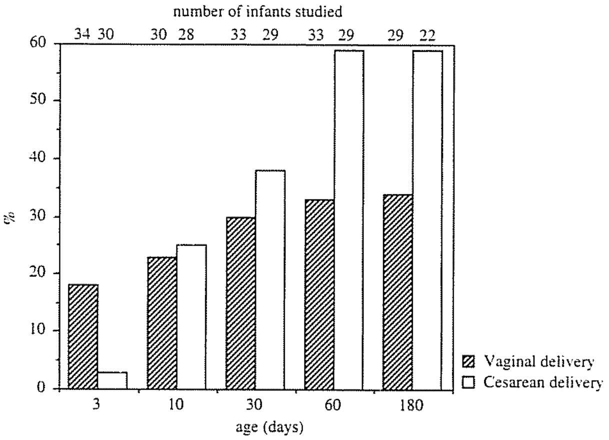

FIG. 2. The percentage of *Lactobacillus*-like bacteria (LLB) colonization in infants aged 3, 10, 30, 60, and 180 days born vaginally and by cesarean delivery.

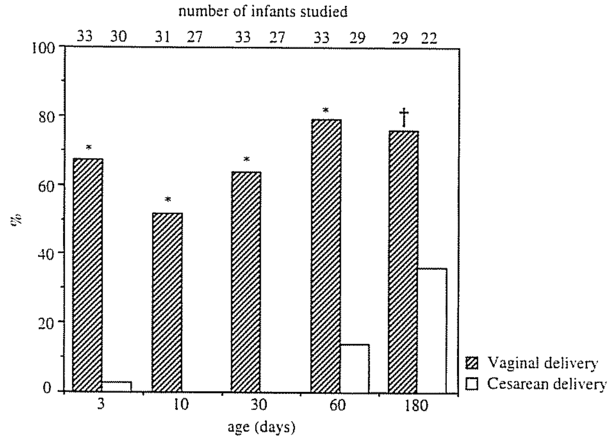

FIG. 4. The percentage of *Bacteroides fragilis* group colonization in infants aged 3, 10, 30, 60, and 180 days born vaginally and by cesarean delivery. *$p < 0.001$, †$p = 0.009$.

LLB counts or in the *Clostridium perfringens* counts between the groups. The amounts of *Bacteroides fragilis* bacteria did not differ between the groups of infants with colonization at 6 months ($p = 0.3$).

Gastrointestinal Signs

Two mothers in both groups did not complete the follow-up sheets. No statistically significant differences were found between the study groups in the scores of abdominal distension, flatulence, or in the amount of colicky crying. Infantile colic, according to the definition of Wessel et al. (20), was detected in three of the VD infants and in none of the CD infants ($p = 0.2$; Fisher's exact test).

DISCUSSION

This study shows that the fecal flora of infants born by CD with prophylactic antibiotics administered to the mother is very different from that of infants delivered vaginally. The greatest differences were seen in the bacteria of the *Bacteroides fragilis* group, which is in agreement with studies in infants born by CD; in earlier studies the duration of follow-up, however, was only 10 to 60 days (1,8,10). In the present study, no permanent colonization with *Bacteroides fragilis* group bacteria was found in the CD group before the infants were 2 months of age. Still, in infants 6 months of age in the CD group, the colonization rate of the *Bacteroides fragilis* group was only half that of infants in the VD group (36% and 76%, respectively; $p = 0.009$). Similarly, Bennet and Nord (8) were unable to detect *Bacteroides* bacteria 3 to 8 weeks after birth in term and preterm infants born by CD.

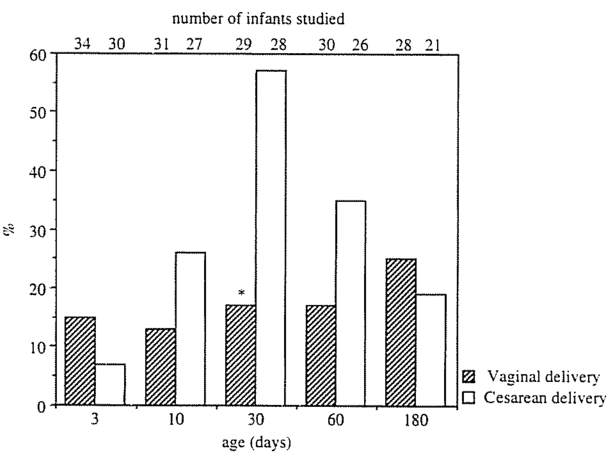

FIG. 3. The percentage of *Clostridium perfringens* colonization in infants aged 3, 10, 30, 60, and 180 days born vaginally and by cesarean delivery. *$p = 0.003$.

We used selective culture media to describe intestinal flora in this study. The counts detected from these selective culture media represent mainly the bacterial genera level but cannot detect the absolute counts of different bacterial genera or species, except those of *Clostridium perfringens,* which were further identified. Nevertheless, this method can identify differences between the delivery groups and the changes taking place in intestinal flora after birth, which was the main purpose of the current study. Furthermore, the main differences in the fecal flora between the delivery groups were found in *Bacteroides fragilis* group bacteria that were detected from the BBE media, which is highly selective for the *Bacteroides fragilis* group (21).

The long-lasting changes seen in the primary gut flora of infants born by CD could be the result of one or both of the two abnormal components of their birth: CD itself or the prophylactic ampicillin administered to the mother 2 hours before the elective CD. Ampicillin is very poorly protein-bound and crosses the placenta readily: maternal and fetal serum ampicillin levels equilibrate within 1 hour after intravenous administration (22). The decline in BLB could be explained by the intravenous ampicillin that the infants were exposed to before delivery, but fecal

TABLE 3. *Median and range of fecal bacterial counts of colonized infants at 3, 10, 30, 60, and 180 days of age*

	Vaginal delivery					Cesarean delivery				
Age (days)	3	10	30	60	180	3	10	30	60	180
Bacteria										
Total	10.6[a]	11.0	11.1	11.3	11.4[c]	10.1	11.1	11.1	11.2	11.1
range	10.1–11.8	9.3–12.1	10.2–11.9	10.1–13.5	9.9–13.5	7.0–11.9	9.5–11.8	10.3–11.7	10.4–13.7	10.1–13.1
n	34	31	33	33	29	30	28	29	29	22
Aerobic enteric	9.6	9.6	9.6	9.5	9.4	9.8	9.9	9.9	9.8	9.6
range	6.7–10.6	8.1–10.8	7.3–10.6	6.6–12.4	7.5–11.7	6.6–10.9	8.8–12.0	7.2–11.0	8.5–13.1	8.1–11.6
n	34	30	33	33	29	29	27	29	29	22
Bacteroides fragilis group	8.7	9.3	9.4	9.5	9.6	6.9	—	—	8.1	8.7
range	4.8–10.2	4.0–10.4	5.5–10.6	5.5–11.1	5.5–12.3	—	—	—	5.5–10.5	6.8–11.2
n	22	16	33	26	22	1	0	0	4	8
Lactobacillus-like	7.8	7.7	8.3	9.3	9.6	6.6	9.4	9.4	8.8	8.9
range	6.4–10.2	6.8–10.4	6.6–9.8	7.4–10.9	5.8–10.1	—	6.5–10.5	7.4–10.3	5.7–10.3	6.8–10.1
n	6	7	10	11	10	1	7	11	18	13
Bifidobacterium-like	10.2[b]	10.7	10.8	10.9	10.9	8.2	10.7	10.8	10.9	10.8
range	7.8–11.1	7.5–11.4	6.4–11.6	8.9–11.8	9.0–12.0	5.1–10.8	5.1–11.8	7.5–11.5	7.2–11.6	8.9–12.1
n	28	30	30	31	27	11	15	24	24	20
Clostridium perfringens	6.7	6.0	7.4	8.2	6.6	7.5	6.9	8.2	6.9	7.0
range	3.8–8.7	4.4–8.0	6.8–9.0	5.8–9.0	5.8–8.5	7.52–7.53	4.1–9.1	4.5–9.5	5.8–10.0	5.5–9.0
n	5	4	5	5	7	2	7	16	9	4

Counts expressed as $\log_{10}$ colony-forming units per gram wet weight of feces.
n, number of colonized infants. *P* are vaginal delivery versus cesarean delivery.
[a] $p = 0.005$.
[b] $p = 0.005$.
[c] $p = 0.03$.

LBL should not decline with ampicillin therapy, at least this is not the case in older children (10 months to 12 years). Also, only a minor decline is detected in fecal *Bacteroides* sp. counts in these older children and the *Bacteroides* sp. counts return to normal stage within only 3 to 6 days after cessation of the ampicillin therapy (23). Thus, it seems unlikely that the long-term changes recorded in the fecal flora of the infants in the CD group in our study could be explained by the administration of ampicillin before delivery. Rather, previous reports imply that this phenomenon is caused by CD itself (1,8). Still, ampicillin could have more profound effects when administered immediately after the birth because the gut is empty, and the drug can select the primary colonizing bacteria.

The changes that took place in the fecal bacterial flora could not be associated with gastrointestinal signs. Any signs were meticulously recorded daily by the mothers for 2 months. In a previous study, *Clostridium perfringens* has been associated with an increased incidence of gastrointestinal signs, such as flatulence, distended abdomen, foul-smelling stools, diarrhea, and blood in stools (12). In the present study, the infants born by CD had a higher colonization rate of *Clostridium perfringens* than the VD group of infants at 1 month (57% and 17%, respectively). Even at this time point, the scores of gastrointestinal signs did not differ between the study groups. The inconsistency of the results between these two studies might be explained by the differences between the study populations. In the previous study most of the infants were treated in the intensive care unit and were preterm infants, whereas in the present study all infants were healthy full-term neonates.

There is great variation among the reports of the predominant bacteria in the fecal flora of breast-fed newborns. Some researchers have found bifidobacteria to predominate and *Bacteroides* to be scarce (2,24,25), whereas others have found the opposite (26,27). However, the predominant bacterium has been classified differently among the studies. Some investigators define the predominant bacterium as the most frequently occurring bacteria (26) and some as the bacterium with the highest counts in fecal samples (28). In the present study, 19 of the 34 VD infants were exclusively breast-fed for 2 months. Among all the VD children, the most frequent bacteria during the first 2 months of life were BLB (85–97%), although *Bacteroides fragilis* group bacteria were also common (52–79%). The highest colony counts were encountered for BLB (10.2–10.9 $\log_{10}$ CFU/g wet weight of feces). The *Bacteroides* colony counts were lower (8.7–9.5 $\log_{10}$ CFU/g). These results agree with those in a recent study from Germany (28).

The present study is the first one to show that the changes in the primary intestinal flora of infants born by CD to mothers who have received antimicrobial prophylaxis last for no less than 6 months after birth, maybe longer. We do not know how long these abnormalities in the fecal flora last on the whole, but the primary quality and quantity of colonization of the gut seems to be critical in the selection process between different genera of bacteria. Some bacteria such as *Bacteroides* are not as easily accessible to the gut of infants delivered the under sterile conditions of CD. Other, perhaps more aerotolerant, bacteria may take over in the intestine and inhibit the subsequent colonization of the gut by *Bacteroides*. This phenomenon is the generally known as interbacterial inhibition (29). Apparently, the bacterial predominances are not readily subject to change under normal domestic conditions after the first months of the colonization process.

Normal intestinal flora has immunostimulatory functions, as has been demonstrated in numerous animal studies (30–32). Mucosal IgA plasma cells are especially scarce in germ-free animals (33). Additionally, when probiotic bacteria belonging to the normal intestinal flora have been administered orally to children in association with diarrhea or mucosal vaccination, an increase in antigen-specific and nonspecific IgA and IgM responses has been detected (34,35). Secretory IgA and IgM are the main humoral mediators of mucosal immunity in cooperation with a variety of innate protective mechanisms. Well-functioning mucosal immunity is a prerequisite for health, because the mucosal surfaces are favored as portals of entry by most infectious agents, allergens, and carcinogens (36). Further research is under way to determine whether the delay found in intestinal colonization in these infants born by CD has any effect on the development of the gut-associated immune system.

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