Reduced Enterobacterial and Increased Staphylococcal Colonization of the Infantile Bowel: An Effect of Hygienic Lifestyle?

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ABSTRACT: The modern Western lifestyle may have altered the composition of the commensal microflora. Here, we investigated the first year's intestinal colonization pattern in 99 vaginally delivered Swedish infants and 17 delivered by cesarean section. Rectal swabs obtained at 3 d of age were cultured for aerobic bacteria and fecal samples obtained at 1, 2, 4, and 8 wk and at 6 and 12 mo of age were cultivated quantitatively for aerobic and anaerobic bacteria. Vaginally delivered infants more often had *Escherichia coli* compared with cesarean section–delivered infants, whereas the latter more frequently carried other enterobacteria, such as *Klebsiella* and *Enterobacter*. Independent of delivery mode, it took 2 mo until most infants were colonized by enterobacteria, traditionally the first colonizers. In contrast, coagulase-negative staphylococci colonized 99% of the infants from d 3 onwards. The poor adaptation of staphylococci to the gut was shown by declining population sizes after some weeks. Dominating anaerobes were initially bifidobacteria and clostridia, whereas *Bacteroides* initially colonized only 30% of vaginally delivered infants and increased very slowly in prevalence. *Bacteroides* colonization was delayed up to 1 y in cesarean section–delivered compared with vaginally delivered infants. Our results show that some “traditional” fecal bacteria are acquired late today especially in cesarean section–delivered infants, probably due to limited environmental circulation. In their absence, skin bacteria like staphylococci have become the first gut colonizers. (*Pediatr Res* 59: 96–101, 2006)

Bacteria establish in the intestinal tract immediately after delivery. *Escherichia coli* and enterococci are present in infant stools within a few days (1,2) and anaerobic bacteria of the *Bifidobacterium*, *Clostridium*, and *Bacteroides* genera within a week (1,2). Other anaerobes are successively established, resulting in a highly diverse microflora at some years of age (3).

The mother's vaginal, fecal, and perineal microbial communities are common sources of infantile bacterial colonization. Infants delivered by cesarean section, who are not ex-

posed to such bacteria, are slower to acquire, e.g. *Bacteroides* and bifidobacteria (4,5). However, environmental sources may contribute significantly to the baby's intestinal colonization (6,7). Thus, infants in developing countries are colonized earlier and by a greater variety of intestinal bacteria than infants in industrialized countries (8,9). Cesarean section–delivered infants in Pakistan acquire *E. coli* almost as early as vaginally delivered ones (8). These findings point to the abundance of faecal bacteria in such environments.

The microflora is a major stimulus for the developing immune system (10), and promotes tolerance to innocuous antigens (11). An altered intestinal microflora may therefore underlie the increased incidence of allergies (12). The present study was initiated to characterize intestinal colonization in a cohort of Swedish infants with high risk of developing allergy, and to determine whether the colonization pattern has changed in the last decades. One hundred sixteen infants were followed with regular sampling and quantitative culture of the intestinal microflora. Colonization by *E. coli* (13) and *Staphylococcus aureus* (14) in part of the cohort and colonization by lactobacilli in the entire cohort (15) has been reported elsewhere. The colonization pattern was related to delivery mode and compared with earlier studies of the intestinal colonization pattern of Western infants.

MATERIALS AND METHODS

Infants, delivery, and hospital care. Swedish infants ($n = 116$) born in 1998–2000 at the Sahlgrenska University Hospital were studied, 99 of whom were delivered vaginally and 17 by cesarean section. They participated in a prospective birth-cohort study, ALLERGYFLORA, investigating the relation between intestinal colonization pattern and allergy development. Informed consent was obtained and the Ethics Committee of Göteborg University approved the study.

Concerning perinatal hygiene, genital wash with chlorhexidine was done before vaginal examinations, which were performed initially and every sixth

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Abbreviations: CFU, colony forming units

hour during labor. In addition, some mothers took a shower with chlorhexidine soap on arrival in the delivery ward.

The average hospital stay was 2.6 d for vaginally delivered and 4.9 d for cesarean section–delivered infants. Eight infants stayed on average 2.2 d at the neonatal ward before transfer to the maternity ward. Rooming in was practiced at the maternity ward, and the infants were almost exclusively handled by the parents and spent the night with their mothers. The maternity and neonatal unit staff practiced regular hand washing and alcohol disinfection.

Six mothers, three of whom underwent cesarean section, received antibiotics in relation to delivery: penicillin G ($n = 1$), cefuroxime ($n = 1$), cefuroxime + metronidazole ($n = 3$), in one case followed by cefadroxil, unknown ($n = 1$).

Most parents were urban professionals living in high-standard houses or apartments, and 89% of the infants had (an) allergic parent(s). Disinfectants are not used regularly in Swedish households. Regular soap and water is most common for personal hygiene, but disposable sanitary towels moistened with disinfectants are often used for cleaning infants, particularly outside the home.

All mothers commenced breast-feeding, but six stopped before 2 mo and thereafter gave commercial formula. Seventy percent of the infants were fully breast-fed for at least 4 mo and 74% were partially breast-fed >6 mo.

Thirty infants (26%) received antibiotics in their first year, 7% ($n = 8$) more than one type: penicillin ($n = 21$), ampicillin/amoxicillin ($n = 13$), trimethoprim/sulphamethoxazole ($n = 4$), erythromycin ($n = 3$), flucloxacillin ($n = 1$), cefuroxime ($n = 1$), ceftibuten ($n = 1$), tobramycin ($n = 1$), and nitrofurantoin ($n = 1$). Eight of the vaginally delivered (8%) and two of the cesarean section–delivered infants (12%) received antibiotics during the first half year, and 22 (22%) and 1 (6%), respectively, during the second half year.

Sampling and culture of the intestinal microflora. The rectal flora was sampled 3 d after delivery using a cotton-tipped swab. The swab was transported to the laboratory in COPAN's transportation medium, and cultured for facultative bacteria within 24 h.

Fecal samples were obtained at 1, 2, 4, and 8 wk and at 6 and 12 mo of age and cultured quantitatively for facultative and anaerobic bacteria. Freshly voided feces were collected by the parents and placed in a sterile Petri dish in a plastic bag with anaerobic atmosphere (AnaeroGen Compact, Oxoid Ltd, Basingstoke, UK), which was kept refrigerated until transported to the laboratory. Using this procedure, counts of relevant bacterial groups were unaltered compared with immediate culture (data not shown). A calibrated spoon of feces was serially diluted in sterile peptone water. Appropriate dilutions were plated on selective and nonselective media (Table 1). From each growth medium, free-lying colonies of different morphology were separately enumerated, Gram-stained, examined in the microscope, and subcultured for purity. The limit of detection was 330 ($10^{2.52}$) CFU (colony forming units)/g of feces.

Table 1. Culture media and conditions of incubation

Medium	Time (d)	Culture conditions	Bacteria atmosphere	Reference
Colombia blood	1	Aerobic	Total aerobes	
Drigalski	1	Aerobic	<i>Enterobacteriaceae</i>	(35)
Staphylococcus agar	2	Aerobic	<i>Staphylococcus</i> spp.	(36)
Enterococcosel agar	1	Aerobic	<i>Enterococcus</i> spp.	(37)
Brucella blood	3	Anaerobic†	Total anaerobes‡	
	3	Anaerobic	Spore formers (<i>Clostridium</i>)§	
BBE agar*	3	Anaerobic	<i>Bacteroides fragilis</i> group	(38)
Beerens agar	3	Anaerobic	<i>Bifidobacterium</i> spp.	(39)
CCFA**	3	Anaerobic	<i>C. difficile</i>	(40)

Rectal swabs and serially diluted fecal samples were cultivated at 37°C on the above-specified media for enumeration of the bacterial groups indicated in the table.

* *Bacteroides* bile esculin agar.

** Cycloserine cefoxitin fructose egg yolk agar.

† Cultured in jars using the BBL GasPak anaerobic system (Becton Dickinson Microbiology Systems, Sparks, NV), using agar plates prerduced for 24 h.

‡ Isolates growing aerobically were not included in the counts.

§ For spore formers (clostridia), feces diluted 1:10 was mixed 1:1 with 99% ethanol and incubated on a shaker at room temperature for 30 min to kill vegetative cells, where after the sample was diluted and plated.

Anaerobes were also subcultured under aerobic conditions (Colombia blood agar plates, 24 h at 37°C), and isolates showing aerobic growth were discarded. However, scanty aerobic growth was accepted for anaerobic Gram-positive rods.

Bacterial identification. The bacterial groups quantified on each plate are shown in Table 1. Enterobacteria were speciated using API20E biotyping (API Systems SA, La Balme les Grottes, Montalieu-Vercieu, France). Staphylococci were identified by Gram-stained appearance and positive catalase reaction. Staphylococci positive in the coagulase test were defined as *S. aureus*, the others as coagulase-negative. Enterococci were identified by esculin hydrolysis and typical Gram-stained appearance.

Bacteroides were speciated by Rapid ID 32A (API Systems). Clostridia, defined as straight Gram-positive or Gram-labile rods with or without spores isolated from alcohol-treated samples cultivated on *Brucella* blood agar, were speciated using Rapid ID32A. *Clostridium difficile* were quantified on selective medium (Table 1) and speciated using Rapid ID32A. Bifidobacteria were identified using a genus-specific PCR (16).

Statistics. Frequencies were compared using Fisher's exact test. Bacterial population counts were compared using Wilcoxon signed-rank test.

RESULTS

Prevalence of facultative bacteria and its relation to delivery mode. It took 2 mo before >90% of the infants had enterobacteria (e.g. *E. coli*, *Klebsiella*, or *Enterobacter*) in their stools. In contrast, all infants harbored staphylococci (coagulase-negative staphylococci and/or *S. aureus*) from 3 d of age. Enterococci also appeared earlier than enterobacteria (Fig. 1).

The effect of delivery mode is shown in Figure 2. Coagulase-negative staphylococci and enterococci colonized vaginally and cesarean section–delivered infants equally early (Fig. 2, *a* and *b*). In contrast, cesarean section–delivered infants less often harbored *E. coli* than vaginally delivered infants over the first 6 mo (Fig. 2c) (3 d, $p = 0.01$; 1 wk, $p = 0.001$; 2 wk, $p = 0.009$; 4 wk, $p = 0.06$; 2 mo, $p = 0.12$; 6 mo, $p = 0.03$).

Enterobacteria other than *E. coli* (mostly *Klebsiella* and *Enterobacter* spp.) were isolated less often from vaginally delivered than from cesarean section–delivered infants (3 d, $p = 0.07$; 1 wk, $p = 0.04$; 2 wk, $p = 0.02$) (Fig. 2d).

Population levels of facultative bacteria. Facultative bacteria colonized at high levels during the first 6 mo but decreased moderately thereafter (6 mo, $10^{9.0}$; 12 mo, $10^{8.3}$

Figure 1. Frequency of colonization by major groups of facultative bacteria in Swedish infants. Enterobacteria (open symbols), staphylococci (filled symbols), and enterococci (hatched symbols) were identified in fecal samples obtained from 116 Swedish infants at different time-points during the first year of life.

Figure 2. Intestinal colonization by facultative bacteria in relation to delivery mode. Colonization of vaginally (filled symbols) and cesarean section-delivered (open symbols) infants by (a) coagulase-negative staphylococci, (b) enterococci, (c) *E. coli*, and (d) other enterobacteria at different time-points during the first year of life. Asterisks indicate significant differences between vaginally and cesarean section-delivered infants (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

CFU/g feces, $p < 0.0001$). The levels differed little between vaginally and cesarean section-delivered infants, provided they were colonized by the bacteria in question. However, cesarean section-delivered infants colonized by enterobacteria other than *E. coli* had higher counts of these bacteria than vaginally delivered infants by 6 mo of age ($10^{8.6}$ versus $10^{7.7}$ CFU/g feces, $p = 0.0006$).

Figure 3 shows the fecal counts of different facultative bacteria in colonized infants, vaginally and cesarean section-delivered infants combined. *E. coli* were 0.5–1 log unit more numerous than other enterobacteria and enterococci throughout the first year. The counts of all three groups decreased significantly after 6 mo of age ($p < 0.0001$ for each comparison) (Fig. 3).

Figure 3. Population levels of different facultative bacteria in culture-positive infants. Population levels (log CFU/g feces) of *E. coli* (filled bars), other enterobacteria (hatched bars), enterococci (dotted bars), and coagulase-negative staphylococci (open bars) in culture-positive infants at different time-points after birth. Both vaginally and cesarean section-delivered infants are included. Mean population levels (bars) and standard deviations are presented. Asterisks above the bars indicate significantly decreased counts compared with the previous sampling occasion (* $p < 0.001$).

Coagulase-negative staphylococci were initially quite numerous but decreased significantly compared with the previous sample from 4 wk until 6 mo of age ($p < 0.0001$ for all comparisons).

Prevalence of anaerobic bacteria and its relation to delivery mode. Anaerobic bacteria were recovered from the feces of 112/116 infants by 1 wk of age. Bifidobacteria dominated initially. Clostridia caught up at 6 mo, whereas *Bacteroides* initially colonized $<1/3$ and increased only slowly in prevalence after 2 mo (Fig. 4).

The effect of delivery mode on anaerobic colonization is shown in Figure 5. Bifidobacteria were more common in vaginally delivered than cesarean section-delivered infants 1 wk after delivery (69% versus 44%, $p = 0.08$) (Fig. 5a). Clostridia were instead significantly more common among cesarean section-delivered infants (1 wk, $p = 0.01$; 2 wk, $p = 0.003$) (Fig. 5b).

Colonization by *C. difficile* increased steadily over the first year (Fig. 5c). Although cesarean section-delivered infants were consistently more often colonized by *C. difficile*, the difference was nonsignificant at all time-points (12 mo, $p = 0.11$).

Bacteroides spp. were found in approximately 30% of the vaginally delivered infants in the first 2 mo, 43% at 6 mo, and 75% at 1 y of age (Fig. 5d). *Bacteroides* first appeared in cesarean section-delivered infants at 2 mo of age, when 1/17 was colonized (Fig. 5d). *Bacteroides* spp. were less frequent in cesarean section-delivered than vaginally delivered infants during the first year of life (1 wk, $p = 0.01$; 2 wk, $p = 0.003$; 4 wk, $p = 0.006$; 2 mo, $p = 0.07$; 12 mo, $p = 0.08$).

Population levels of anaerobic bacteria. Anaerobic population counts increased from $10^{10.1}$ to $10^{10.3}$ CFU/g between 1 wk and 1 y of age ($p = 0.01$, only colonized infants included). The levels were equal in vaginally and cesarean section-delivered infants.

When present, bifidobacteria and *Bacteroides* were most numerous (Fig. 6). Clostridial spores amounted to 10^5 – 10^6 CFU/g feces. Vegetative clostridial bacterial levels could not be determined due to lack of selective media. However,

Figure 4. Frequency of colonization by major groups of anaerobic bacteria in Swedish infants. Bifidobacteria (open symbols), clostridia (hatched symbols), and *Bacteroides* spp. (filled symbols) were identified in fecal samples obtained from 116 Swedish infants at different time-points during the first year of life.

Figure 5. Intestinal colonization by anaerobic bacteria in relation to delivery mode. Colonization of vaginally (filled symbols) and cesarean section-delivered infants (open symbols) by (a) bifidobacteria, (b) clostridia, (c) *C. difficile*, and (d) *Bacteroides* at different time-points during the first year of life. Asterisks indicate significant differences between vaginally and cesarean section-delivered infants (* $p < 0.05$, ** $p < 0.01$).

Figure 6. Population levels of different anaerobic bacteria in culture-positive infants. Population levels (log CFU/g feces) of *Bacteroides* (filled bars), bifidobacteria (hatched bars), spore-forming clostridia (dotted bars), and *C. difficile* (open bars) in culture-positive infants at different time-points after birth. Both vaginally and cesarean section-delivered infants are included. Mean population levels (bars) and standard deviations are presented.

clostridia often grew in high counts (10^8 – 10^{10} CFU/g feces) on the nonselective *Brucella* blood agar plate.

Influence of allergic heredity on intestinal colonization pattern. The cohort was selected to have high risk of developing allergies and most infants had (an) allergic parent(s). Thirteen infants had no allergy in the family. Their colonization pattern did not differ from the majority, except that they were significantly less often colonized with clostridia at 1 y of age ($p = 0.014$).

Influence of antibiotics on intestinal colonization pattern. Colonization in the first 6 mo was compared between infants receiving, or not receiving antibiotics during this period. Colonization at 12 mo was compared between infants receiving, or not receiving antibiotics between 6 and 12 mo of age.

Antibiotic treated infants less often had clostridia than nontreated infants (1 wk, $p < 0.10$; 2 wk, $p < 0.05$; 1 mo, $p = 0.19$; 6 mo, $p < 0.01$; 12 mo, $p = 0.11$). Instead, during the first 2 mo of life, they tended to more often have *E. coli* (3 d, 1 mo, $p < 0.10$; 2 mo, $p < 0.05$) and *S. aureus* (3 d, $p = 0.15$; 1 wk, $p = 0.11$; 2 wk, $p < 0.10$; 2 mo, $p = 0.17$) than nontreated infants.

Infants to mothers treated with antibiotics around delivery less often had *E. coli* than other infants (3 d, 1, 2 and 4 wk, $p < 0.05$; 2 mo, $p < 0.01$; 6 mo, $p = 0.05$). They also tended to less often have bifidobacteria (1 mo, $p < 0.05$) and clostridia (2 mo, $p < 0.05$; 6 mo, $p < 0.10$).

DISCUSSION

We here describe the longitudinal intestinal colonization pattern during the first year of life in more than 100 infants born in Sweden by the end of the 20th century. To our knowledge, this is the largest birth cohort studied with respect to the establishment of the commensal intestinal microflora. Although differences in methodology make direct comparisons with studies from the 1970s and 1980s difficult, there are indications that infantile colonization might have changed in the last decades in Sweden.

A striking observation was the ubiquitous colonization by coagulase-negative staphylococci throughout the first year of life. These bacteria, not previously regarded as intestinal commensals, initially reached as high population counts as typical fecal bacteria like *Klebsiella*, *Enterobacter*, and enterococci. *S. aureus* was isolated from three out of four infants in this birth cohort, as previously reported (14). Staphylococci were infrequent in the bowel flora of Western infants investigated in the 1970s (2,17,18). In the 1980s, *S. epidermidis* were isolated from 30 to 70% of 1-wk-old Swedish infants (9,19,20), suggesting that they had become more common. Anecdotal, increased staphylococcal colonization between 1975 and 1995 was described in France (21).

Staphylococci are more common in breast-fed than bottle-fed infants (20,22). The high prevalence of staphylococci might relate to the very high breast-feeding rate. However, the few bottle-fed infants were equally often colonized by *S. aureus* as the breastfed majority (Lindberg *et al.*, manuscript in preparation). We suggest that lack of competition from “professional” gut bacteria may explain the expansion of staphylococci in the infantile gut flora. “Classical” fecal bacteria like *E. coli* appeared late in the present cohort (13). Only 56% of vaginally delivered infants were colonized by 1 mo of age, compared with 60–90% in the 1980s (19,20,23,24) and 80% in the 1970s (25) in Sweden. *E. coli* thrives only in the intestines of man and animals and is spread by fecal contamination. In the 1970s, around a quarter of vaginally delivered infants acquired *E. coli* from their mothers; the rest were colonized by strains spread by the hospital staff (25). Today, short hospital stays, rooming-in, and better hospital hygiene might have reduced such exposure. Other enterobacteria that commonly colonize newborn infants, such as *Klebsiella* and *Enterobacter* spp., rarely derive from the mother but spread in maternity and neonatal wards (3,6). Only 18% of the vaginally delivered infants examined here had such bacteria at 1 wk, which can be compared with >40% in a previous study performed by us in the 1980s (8). This confirms the declining colonization of Swedish infants by hospital bacteria.

Enterococci are also typical fecal bacteria, but they are more resistant than others to hygienic measures (26). Enterococci were isolated by us as often as reported previously (20),

and cesarean section–delivered infants were equally early colonized as those delivered vaginally. This shows that some bacteria resist hygiene better than others. Among these are clostridia, which form spores that resist disinfectants and occur in abundance in the environment (27). Hence, clostridia dominated the early anaerobic flora in cesarean section–delivered infants.

Bifidobacteria may be transferred from the mother during delivery (7) and they were more frequent in vaginally compared with cesarean section–delivered infants in the first week. However, the cesarean section–delivered infants soon acquired bifidobacteria from yet undefined sources. Bifidobacteria are relatively tolerant to ambient oxygen, which may facilitate their spread (3). As most infants were colonized by bifidobacteria, they seem not to have been reduced by the contemporary lifestyle.

Spread of fecal bacteria appears to have diminished not only in the modern hospital, but also in families. Thus, cesarean section–delivered infants had not yet caught up with vaginally delivered infants with respect to *E. coli* colonization by 6 mo of age. In earlier studies from Western countries, this process seems to have occurred faster (28,29), and in Pakistan, practically all cesarean section–delivered infants acquire *E. coli* within a week (8).

Bacteroides were also isolated less often than in earlier Swedish studies (5,20), and many cesarean section–delivered infants had not yet acquired *Bacteroides* by 1 y of age. In a recent study from Finland (4), *Bacteroides* was delayed in cesarean section–delivered infants up to 6 mo of age and in studies from Western countries in the 1980s at least during the first weeks of life (5,28). By contrast, in Nigeria, both vaginally and cesarean section–delivered infants harbor *Bacteroides* within a week in most cases (30). We regard the delayed *Bacteroides* colonization as another indication of limited circulation of fecal bacteria today.

As a more and more complex anaerobic flora develops the facultatives are suppressed (3). This was manifested by declining counts of enterobacteria and enterococci after 6 mo of age. The counts of coagulase-negative staphylococci dropped faster and more strikingly, which was also observed for *S. aureus* (14). Their inability to compete with other bacteria in a microflora of increased complexity distinguish them from more “professional” intestinal bacteria. The presence of staphylococci in almost all 1-y-old infants indicates a slow acquisition of a highly complex microflora in Swedish infants today. Another sign of this is that *C. difficile* isolation rates increased steadily up to 12 mo of age. *C. difficile* is common in the microflora of young infants, but firmly suppressed by the anaerobic flora in adults (27). In a Swedish study performed some 15 y ago, colonization by *C. difficile* peaked at around 6 mo of age, where after it decreased (31).

Antibiotics influence intestinal colonization (5,19) and a quarter of the infants examined here received antibiotics in their first year. However, because treated infants tended to have more *E. coli* and *S. aureus*, but less clostridia, antibiotic treatment could neither explain low colonization rates of *E. coli* and *Bacteroides*, nor the abundance of coagulase-negative staphylococci. *S. aureus* was also very prevalent in infants

who had not received antibiotics (unpublished observations). Moreover, although antibiotic utilization increased steadily in Sweden until the mid-1990s, it decreased thereafter, especially in children. For instance, consumption of penicillin and macrolides in the age group 0–6 y is similar today compared with 1974 (32). We do not regard antibiotic treatment of infants as a significant cause of delayed colonization by fecal bacteria.

Six mothers received antibiotics during or after delivery. Their infants acquired *E. coli* very late and tended to have less clostridia and bifidobacteria. As three of these infants were delivered by caesarean section, the contribution of maternal antibiotics is difficult to judge. However, these few infants could only minimally affect the colonization pattern of the cohort. Therefore, maternal antibiotics could also not explain the slow colonization by typical faecal bacteria.

The present study suggests that Gram-negative bacteria (enterobacteria and *Bacteroides*) seem to have decreased in the last decades, whereas Gram-positive bacteria (enterococci, bifidobacteria, clostridia and staphylococci) remain high or have even increased. As the intestinal microflora is a common source of bacteria causing extraintestinal infections, it is interesting to note that staphylococci are the most common cause of neonatal septicemia today (33). Furthermore, the intestinal microflora is the major drive for the gut immune system (10). Inasmuch as Gram-positive and Gram-negative bacteria induce partly different mediators when interacting with the innate immune system (34), a changed colonization pattern caused by increased hygiene may have global effects on the developing infantile immune system.

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