Postnatal Development of Intestinal Microflora as Influenced by Infant Nutrition[1,2]

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Abstract

The postnatal period of a new human being is characterized, from the microbiological point of view, by the formation of a new ecosystem: the microflora of the human gut. In adulthood a number of barriers exert a potent selective action on bacteria arriving from the mouth, but in the very first stage of our life, these barriers are kept at a very low level, temporarily allowing penetration into the gut of bacteria that are not really believed to be ''gut related.'' Moreover, type of delivery (natural vs. cesarean) and feeding (breast vs. bottle feeding) play dramatic roles in determining the microflora composition. In the last decade a number of articles have reported results on neonates' microflora obtained by means of culture-independent analysis. Data obtained by means of these techniques are in agreement with those produced by selective media, but they also provide some new insights about the presence of anaerobic bacteria. The focus of this article is to update knowledge on infants' microflora during the first 6 mo of life. J. Nutr. 138: 1791S–1795S, 2008.

Introduction

Birth is an exciting moment for the newborn, parents, and relatives, but it is also exciting for the microbial ecologist. The postnatal period of a new human being offers a great opportunity to study, from the very beginning, the formation of a new ecosystem: the microflora of the human gut. At birth the fetus is sterile, and the first encounter with the microbial world begins during delivery. In adulthood a number of barriers exert a potent selective action on bacteria arriving from the mouth, but in the very first stage of life these barriers are at a very low level and temporarily allow penetration into the gut of bacteria that are not really believed to be gut related.

Moreover, type of delivery (natural vs. cesarean) and feeding (breast vs. bottle feeding) play dramatic roles in determining the microflora composition. The relation between microflora composition and type of feeding offers an opportunity to develop a nutritional strategy to favor the most efficient microflora in terms of health protection.

Since the beginning of the previous century, efforts have been devoted to describe bacterial succession in the gut of newborns by means of microbiological analysis of infants' stools. Plate counts on selective media have yielded what is believed to be solid knowledge on the formation of this ecosystem [for a review see Mackie et al. (1)]; however, in the last decade, a number of articles have reported results on neonates' microflora obtained by means of culture-independent analysis, such as fluorescent in situ hybridization (FISH)[3] or denaturing gradient gel electrophoresis (DGGE), 16S RNA cloning and sequencing, as well as real-time PCR. Data obtained by means of these techniques are in agreement with those produced by selective media, but they also provide some new insights about the presence of anaerobic bacteria.

The focus of this article is to update knowledge on infants' microflora during the first 6 mo of life by reviewing data obtained by culture-independent techniques. Microflora of preterm newborns is not considered here because it was recently reviewed (2).

The very first days

During birth, bacterial colonization of a previously germ-free gut begins. Type of delivery is crucial in selecting the first colonizers. Naturally delivered babies experienced a period of 2–3 d in which, as a consequence of the low selective potential of their stomach and small bowel, bacteria invading and reproducing within the gut belong to aerobic species such as *Enterobacteriaceae*, streptococci, and staphylococci. These bacteria, arriving from the external environment, belong to species with a pathogenic potential, and therefore, it might seem that they would not be the best choice for the health of neonates. However, the metabolisms of these bacteria are believed to be positive factors in preparing the path to a beneficial enteric flora (3). According to data obtained by means of classical microbiological techniques, bifidobacteria, lactobacilli, and other anaerobic bacteria appear to reach the gut after 2–3 d.

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[3] Abbreviations used: DGGE, denaturing gradient gel electrophoresis; FISH, fluorescent in situ hybridization; Q-PCR, quantitative polymerase chain reaction.

Newborns delivered by cesarean section seem to have a reduced number of bacteria compared with those naturally delivered infants; moreover, the appearance of bifidobacteria seems to be delayed for up to 6 mo of life (4).

Environmental sources of the bacteria that are first to arrive into the gut of newborns are believed to be the vagina, skin, and feces of the mother, providing an inoculum that is a mix of intestinal and nonintestinal adapted species.

In vaginal delivery, the same serotypes of *Escherichia coli* were found both in babies immediately after birth and in their mothers' feces, strongly suggesting that microbes from mothers' feces contaminate infants (5).

Vaginal microflora was also shown to be a source of first colonizers in a study reporting that the gastric content of 5- to 10-min-old babies was similar to that of their mothers' cervix (6).

Cesarean delivery demonstrates that exposure to environmental bacteria (equipment, air, other infants, nursing staff) can be more significant than the mother's bacteria in inoculating neonates, thus delaying the onset of true intestinal bacteria.

After contamination related to delivery, environmental, oral, and skin microbes from the mother provide the major source of bacteria for the newborn via modes of transfer such as suckling, kissing, and caressing.These bacteria have been shown to be able to create a reduced environment favorable to anaerobic bacteria, which colonize the gut at the end of wk 1 of life, taking over from the aerobic bacteria.

An additional source of bacteria for the breast-fed neonates is mother's milk, which contains up to 10^9 microbes/L in healthy mothers (7). The most frequently encountered bacterial groups include staphylococci, streptococci, corynebacteria, lactobacilli, micrococci, propionibacteria, and bifidobacteria, and the authors suggest that these bacteria originate from the nipple and surrounding skin as well as the milk ducts in the breast.

It has recently been suggested that human breast milk could be a relevant source of lactobacilli for newborns (8,9); in addition to plating, authors have used DNA fingerprints to identify the same *Lactobacillus* strains in the milk of mothers and in fecal samples of their babies. A distinct ecological niche is formed by the oral cavity of the neonate and the skin surrounding the mother's nipple as well as her nipple itself.

Quite recently some changes in the composition of these first colonizers have been reported, and these have been linked to more stringent hygienic conditions of delivery. Skin-derived staphylococci are becoming more abundant than fecal Enterobacteriaceae (10,11).The picture painted by classical microbiological analysis is that an initial colonization of environmentally derived bacteria prepares the gut to host truly intestinal anaerobic species. Molecular ecology is now making it possible to identify some of the participants in this process.

In addition to detection of bacterial groups by cultivation, DGGE analysis has made it possible to identify the presence of clostridia in these very first days (3), suggesting that "the first dominant colonizer of a baby can be a member of the clostridia" (3).Even if this observation has been limited to an extremely reduced number of subjects, the possibility that during the first 3 d of life the gut environment is already sufficiently anaerobic to support the growth of clostridia is puzzling for the microbial ecologist, and it is challenged by conflicting results. A Korean group (12) used 9 independent 16S RNA libraries obtained by fecal samples of a single neonate at d 1, 3, and 6; on d 1 of the life of the infant, they found mainly aerobic or facultative species such as *Enterobacter, Lactococcus lactis, Leuconostoc citreum,* and *Streptococcus mitis,* with *L. lactis* representing the largest group. On d 3 of life, *Enterobacter, Enterococcus faecalis, Escherichia coli, S. mitis,* and *Streptococcus salivarius* were present, replaced on d 6 of life by *Citrobacter, Clostridium difficile, Enterobacter* sp., *Enterobacter cloacae, and E. coli.* Furthermore, DGGE analysis (13) of the microflora of Japanese neonates showed the presence, during the very first days and before the arrival of gram-positive cocci, of isolates belonging to *Pseudomonas*.

These conflicting results highlight the need to develop additional molecular tools for investigating intestinal microbial ecology.

The bifidobacteria days

The classical view of bacterial succession in neonates states that by 1 wk of age, vaginally delivered, breast-fed infants had a microflora largely dominated by bifidobacteria (14), whereas bottle-fed babies had a more heterogeneous composition of their flora.

This pattern has recently been confirmed by molecular analysis such as FISH (15) and real-time PCR (16).

However, molecular methods have shown that another additional anaerobic bacterial group is to be considered as "dominant" after the very first days of life, at least in breast-fed babies: *Ruminococcus.* The presence of this genus has been detected by DGGE, although random cloning of 16S RNA has shown that ruminococci are present at the same level as bifidobacteria (3,17).

Recently (G. Coppa, L. Morelli, S. Soldi, O. Gabrielli, A. Carlucci, unpublished data), breast-fed neonates were recruited and grouped according to the typology of the breast milk oligosaccharide composition, and 39 infants had their feces sampled on d 30 of life. Ruminococci were found, by means of species-specific PCR, in 25 of the 39 subjects examined, suggesting that this genus could be nearly as widespread as bifidobacteria in breast-fed babies.

It is also interesting to note that ruminococci were found to be positively affected by oligosaccharides, at least in animal models (18). Because it is well known that breast milk is an enormous source of prebiotic compounds (19), it will be of interest to measure the impact of the complex sugars on these bacteria, either in breast-fed babies or in infants fed with formula enriched with prebiotic fibers.

The complete role of ruminococci in protecting the health of babies is far from being understood, although we have at least 1 clue: *Ruminococcus* is recognized to have an important protective effect on the host because it produces ruminococcin A, a bacteriocin that can inhibit the development of many species of *Clostridium* (20). Quite interestingly, 1 of the most striking differences between microflora of bottle- and breast-fed babies is the low presence of clostridia in the latter group (14).

DNA of ruminococci has been detected in breast-fed babies, and molecular biology techniques have made it possible to detect the presence, mainly in formula-fed babies, of members of the genus *Desulfovibrio* (16,21). Members of this genus are the predominant sulfate-reducing bacteria in adults, and these organisms are of particular interest because of their potential link with inflammatory bowel disease. Until now it was thought that they might not be able to colonize the gut until late childhood. This genus is not easy to enumerate reliably using culturing methods, and it is often ignored in studies on the colonic microflora.

In addition, a recent article has reported the detection, even if at low level, of methanogenic bacteria in fecal DNA samples from 6 children under the age of 1 y, the youngest being only 4 mo old (21).

The problem of enumeration of anaerobic bacteria is also indicated by results reported by Harmsen et al. (22), which

showed that FISH analysis resulted in a number of *Bacteroides* cells equal to the number of bifidobacteria in formula-fed infants, whereas in the culture-based studies, the *Bacteroides* numbers remained 100- to 1000-fold lower. The authors conclude that "This clearly indicates that there is a problem in culturing this group of anaerobic bacteria" (22). Interestingly, in the same article it has been reported that enumeration with FISH indicated that a high number of bifidobacteria were associated with a low *Bacteroides* count and vice versa.

FISH analysis with specifically designed probes (22) has also made it possible to detect a relevant amount (calculated as fluorescent percentage) of the anaerobic bacteria of the *Coriobacterium* group (*Collinsella* and *Atopobium*) in 6 bottle-fed neonates aged 12 d; in contrast, in 6 breast-fed neonates, the presence of these bacteria was negligible.

The largest study on intestinal flora of neonates performed using a molecular tool such as quantitative PCR (Q-PCR) has been published by a Dutch group (23). Fecal samples were collected at 1 mo of age from 1034 neonates, representing all possible delivery and feeding alternatives. Five bacterial groups and a bacterial total load were counted by means of Q-PCR. Results confirmed the strong impact of cesarean section on intestinal microflora (less bifidobacteria and widespread presence of *Clostridium difficile*).

Q-PCR has also been used to enumerate bifidobacteria and lactobacilli in babies aged 28–90 d (24,25). Even if these 2 studies are intervention ones, they report relevant data on the control groups of breast- and bottle-fed infants. Bifidobacteria were detected using Q-PCR and FISH in 10 babies, and results confirm the lower presence of bifidobacteria in babies fed with a formula lacking prebiotic substances and also suggest a different species distribution in these babies.

In regard to lactobacilli, results strongly confirm the massive presence of *L. acidophilus* followed by *L. casei* and *L. paracasei*, as already shown by Morelli et al. (26) in breast-fed infants, whereas babies fed with a standard formula harbored mainly *L. delbrueckii* and *L. reuteri*; *L. acidophilus*, however, was also present in these babies but at a lower level.

Taken together, all these observations suggest that a more in-depth characterization of the anaerobic flora of infants is worthwhile to understand the role of these bacteria during the first weeks of life. Moreover anaerobic bacteria detected by molecular biology have shown that the 2 types of feeding cause differences in gut flora composition never before suspected.

Approaching the weaning

After the first 2 wk of life, it seems that a quite stable, feeding-related (breast vs. formula milk) microflora is established and stably maintained. Quite interestingly, supplementation with formula milk induces a rapid shift in bacterial pattern of a breast-fed baby (3).

The environment provided by the family seems to have a dramatic impact on microflora composition during this period. DGGE profiles obtained after 1 and 3 mo of life of 5 babies, showed that 3–7 of their bands were comigrating with those present in their parents' profile, suggesting the presence of the same bacterial groups in different subjects (17).

A study on prebiotic supplementation of formula-fed babies (aged 28–90 d) showed (26) an increase in the load of *Lactobacillus* species in the control group of breast-fed babies as well as in the prebiotic-supplemented group but not in subjects given standard formula, suggesting that human milk and prebiotic fibers are not only bifidogenic but could also support the growth of lactobacilli.

Weaning

Quite curiously, microflora composition during weaning has only recently been investigated by means of classical (27) and molecular techniques (28). Both studies showed a substantial stability of bifidobacteria, which confirmed a previous study (29). Amarri et al. (27) showed that lactobacilli and vancomycin-insensitive lactobacilli increased significantly from 120 to 210 d of age and then decreased. The same authors measured the gut permeability and immune markers, detecting changes of the eosinophil cationic protein and sIgA in stool samples during weaning. The observed reduction in fecal eosinophil cationic protein suggests a decrease in gut permeability and a possible reinforcement of gut mucosa integrity. The reduction of sIgA during the early weaning period has been attributed by these authors to a decline of the immune contribution provided by breast milk as suckling declines and to a reduction in weaning-elated stress.

Intestinal microflora composition and allergy

The potential role played by intestinal bacteria, including probiotics, in reducing the risk of developing allergy has been addressed by a number of articles [for a review see Kalliomäki and Isolauri (30)].

Some of these articles have tried to establish an ecological link between allergy and the microflora composition. Studies

TABLE 1 Summary of results obtained by means of culture-independent analysis on fecal samples of full-term neonates

References	Method of analysis (*n* subjects analyzed)	Notes	Results
15	FISH (12)	6 breast-fed and 6 bottle-fed	Detection of *Atopobium, Collinsella,* and *Bifidobacterium*
3	DGGE/TTGE (2)		First article reporting a long-lasting observation of intestinal flora by means of culture-independent techniques
17	DGGE/TTGE (5)		Similarities between parental and neonate microflora
16	Q-PCR (40)	The average age of the children was 11.5 mo (3 wk to 24 mo)	Detection of *Desulfovibrio*
12	Cloning and sequencing (1)	Sampled during d 1, 3, and 6	
13	DGGE/TTGE (9)	Sampled for 2 mo	*Pseudomonas* detected during the first days
24	FISH (10) and QPCR (10)	Data from an intervention study	Detection of bifidobacteria
28	DGGE/TTGE (11)	Weaning	Bifidobacteria stable and ruminococci increase during weaning
23	QPCR (1032)		5 bacterial groups and total bacteria monitored
21	QPCR (40)	Children aged up to 10 y	Presence of methanogens and sulfate-reducing bacteria

have been done in Japan (31,32), in Europe (33), and in New Zealand (34). However, caution must be used before it can be concluded that a real link does exist between microflora composition in the early days of life and the onset of allergy in later life. Clinical trials where large numbers of subjects have been enrolled have not confirmed such a link (33) when infants were recruited perinatally in Göteborg ($n = 116$), London ($n = 108$), and Rome ($n = 100$). The conclusions of these studies were that data obtained in these 3 cohorts after 18 mo of life do not support the hypothesis that sensitization to foods or atopic eczema in early life is associated with the lack of any particular culturable intestinal commensal bacteria. Identification of the microbial group required for protection from allergy remains to be identified.

Definitely, the need for culture-independent techniques to assess the presence of various bacterial groups in intestinal microflora has been pointed out by results obtained using molecular identification of the colonies grown in selective media, which showed that this procedure is insufficiently selective and unsuitable for quantitative analyses (15).

The number of subjects investigated by means of culture-independent techniques is still very limited (**Table 1**), but data obtained so far are providing a wealth of information on the whole microbial ecology of the postnatal colonization of the human gut.

Furthermore, the easier approach provided by molecular biology is to classify lactobacilli and bifidobacteria isolated from the stool of infants taxonomically by allowing a clear view of the composition of the microflora at the species level; this will help to establish the role of each single bacterial component of the microflora of neonates (24–26).

It is prudent, however, to point out again that, at the moment, the number of subjects studied by means of molecular biology, although growing, is still limited; moreover, molecular biology techniques also have some bias; just as an example, the first articles reporting DGGE analysis of the stool of infants clearly underestimated the presence of lactobacilli (3,17), which are generally counted by selective plating.In addition, analytical methods based on DNA encoding for 16S RNA are unable to discriminate between living and dead cells; DNA extraction methodology can underestimate the number of bacterial spores, etc.

It must also be emphasized that the only available data on microbiota composition of neonates have been obtained using stool samples; the microflora composition of the upper part of the gut is thus largely unknown.

All the above suggest that our knowledge on postnatal microbial development is far from complete and that the next research efforts should be geared to illuminate our knowledge of this field, including provision of a timeline for the presence of strictly anaerobic (unculturable) bacteria.

New data on the accurate composition of infant microflora will allow improved nutritional strategies and a better comprehension of the host-bacterial relations. In addition, as a consequence of the availability of new diagnostic techniques, a revision of the actual composition of the microflora of breast-fed babies, which is believed to be the "golden standard," is probably mandatory.

Other articles in this supplement include references (35–44).

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